STUDIES OF THE HYDROXYLATION OF CHOLESTEROL IN ENDOCRINE TISSUES

by

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SUMMARY

A brief summary of the work described in this Thesis is given below:-

(a) A rapid and sensitive assay of cholesterol side-chain cleavage is described.

(b) Using this technique, the enzyme system responsible for the conversion of cholesterol to pregnenol-:one in bovine adrenal cortex was investigated.

(c) The formation of pregnenolone from cholesterol was found to be inhibited by carbon monoxide. This inhibition was reversed by light. The photochemical action spectrum for this light-reversal had a maximum at 450mμ.

(d) The nature of the intermediates between cholesterol and pregnenolone was investigated.

(e) The cholesterol side-chain cleavage system was resolved into three protein fractions - a fraction containing cytochrome P-450, a non-haem iron protein, and an NADPH-diaphorase. The latter two proteins constitute an NADPH-cytochrome P-450 reductase.

Abbreviations used in the Text

The following standard abbreviations will be used throughout the text:-

$NADP^+$	-	nicotinamide adenine dinucleotide phosphate
NADPH reduced	-	" " " "
NAD^+	-	nicotinamide adenine dinucleotide
NADH reduced	-	" " "
ATP	-	5'- adenosine triphosphate
ADP	-	5'- adenosine diphosphate
AMP	-	5'- adenosine monophosphate
3',5'-AMP	-	3',5'- adenosine cyclic monophosphate
FAD	-	flavine adenine dinucleotide
FMN	-	flavine mononucleotide
G-6-P	-	D-glucose 6-phosphate disodium salt
G-6-P dehydrogenase	-	D-glucose 6-phosphate dehydrogenase
T.L.C.	-	Thin-layer chromatography
G.L.C.	-	Gas-liquid chromatography
PPO	-	2,5-diphenyloxazole
POPOP	-	1,4-bis-[2-(5-phenyloxazolyl)]-benzene

1. GENERAL INTRODUCTION

(1) Occurrence and Biosynthesis of Cholesterol

Sterols are a group of C_{27-29} secondary alcohols of animal and plant origin which differ from common alcohols in being crystalline solids of melting points in the range 100-200°C. In higher animals the only major sterol is cholesterol. Its structure was elucidated by the classical work of Wieland, Windaus, Diels, Rosenheim and King, which is reviewed by Fieser and Fieser (1959).

Cholesterol is present in all tissues and all cells. It does not occur free to any significant extent because of its highly hydrophobic nature, but instead occurs bound to lipoprotein complexes which are an integral part of cell membranes. It is found both free and esterified at the 3β-position with various fatty acids. For example, in liver the predominant fatty acid esterified to cholesterol is generally oleic acid, followed by palmitic acid. The short chain fatty acids are not found esterified with cholesterol to any great extent. Cholesterol ester metabolism is reviewed by Goodman (1965).

The turnover rate of cholesterol varies greatly in different tissues. For example, liver, plasma and adrenal cortex have rapid turnover rates, whereas adult brain has a very low turnover rate (Avigan, Steinberg and Berman, 1962). All tissues with the exception of adult brain

have the ability to synthesise cholesterol, although this property is possessed by developing brain. Certain tissues such as liver, adrenal cortex and gonads are also capable of cholesterol degradation. Much of the turnover of cholesterol is accountable however, in terms of exchange of tissue cholesterol with cholesterol in the plasma. Generally, tissues with a rapid turnover rate of cholesterol have a relatively high percentage of cholesterol in the esterified form, such as adrenal cortex, whereas brain, with a very low turnover rate, has essentially no cholesterol in the esterified form. The significance of this, however, is not understood at present. Within the cell, cholesterol is found in all the membranous structures - the cytoplasmic membrane, mitochondria, endoplasmic reticulum and nuclear membrane and also in lipoprotein complexes in the cytoplasm, which contain most of the esterified cholesterol. Little is known of the exchange of cholesterol between these structures because of the difficulty of designing experiments to study this problem, although the final stages of cholesterol synthesis take place in the endoplasmic reticulum. It is obvious therefore that there are many metabolic pools of cholesterol, both within the tissues of the body as a whole and within the individual cells themselves.

Cholesterol Biosynthesis

Although a portion of dietary cholesterol is readily absorbed by higher animals, it is not an essential component of their diet, and the fact that most animals can synthesise their sterols from smaller carbon compounds has long been known. The elucidation of the pathway of cholesterol biosynthesis was accomplished due to the studies of Bloch and co-workers (reviewed by Bloch, 1954) and of Cornforth and associates (Cornforth, Hunter and Popjak, 1953 and Cornforth, Gore and Popjak, 1957). All the 27 carbon atoms of cholesterol are derived from the carbon atoms of acetate. Briefly, the first stages in the synthesis of cholesterol are concerned with the formation of an isoprenoid unit, isopentenyl pyrophosphate, which is polymerised to the triterpene squalene. Cyclisation of this gives the C_{30} sterol lanosterol which undergoes re-arrangement and loss of 3 carbon atoms to form cholesterol. The events leading to the synthesis of squalene do not require oxygen, but the cyclisation of squalene and some of the succeeding reactions require aerobic conditions.* This is because these are mixed-function oxidase reactions, requiring molecular oxygen and NADPH (Mason, 1957). The synthesis of cholesterol has much in common with that of terpenoids such as the carotenes and natural rubber. An excellent review of cholesterol synthesis is that of Popjak and Cornforth (1960).

* Some bacterial systems are known which effect the cyclisation of squalene anaerobically.

(2) Degradation of Cholesterol

As mentioned previously, certain tissues are capable of degrading cholesterol, namely the liver, adrenal cortex, placenta and gonads. Quantitatively the most important catabolic pathway is the degradation to bile acids and neutral sterols which occurs in the liver. Of great physiological importance however, is the catabolism of cholesterol to the steroid hormones which takes place in the endocrine tissues.

The first step in the catabolism of cholesterol to the bile acids is hydroxylation at the 7α-position. The enzyme system responsible for this reaction occurs in the endoplasmic reticulum. Thus, the initial attack is on the ring structure of the cholesterol molecule. On the other hand, the initial event in cholesterol metabolism to the steroid hormones is cleavage of the cholesterol side-chain to form the C_{21} steroid, pregnenolone. The enzyme system catalysing this reaction occurs in the mitochondria in each of the tissues involved. However, subsequent degradation of the cholesterol side-chain in liver to form the C_{24} bile acids appears to occur in the mitochondria, whereas in endocrine tissues, most of the subsequent reactions take place on the ring structure and many occur in the endoplasmic reticulum. Thus, there are fundamental differences in the two initial reactions, each of which

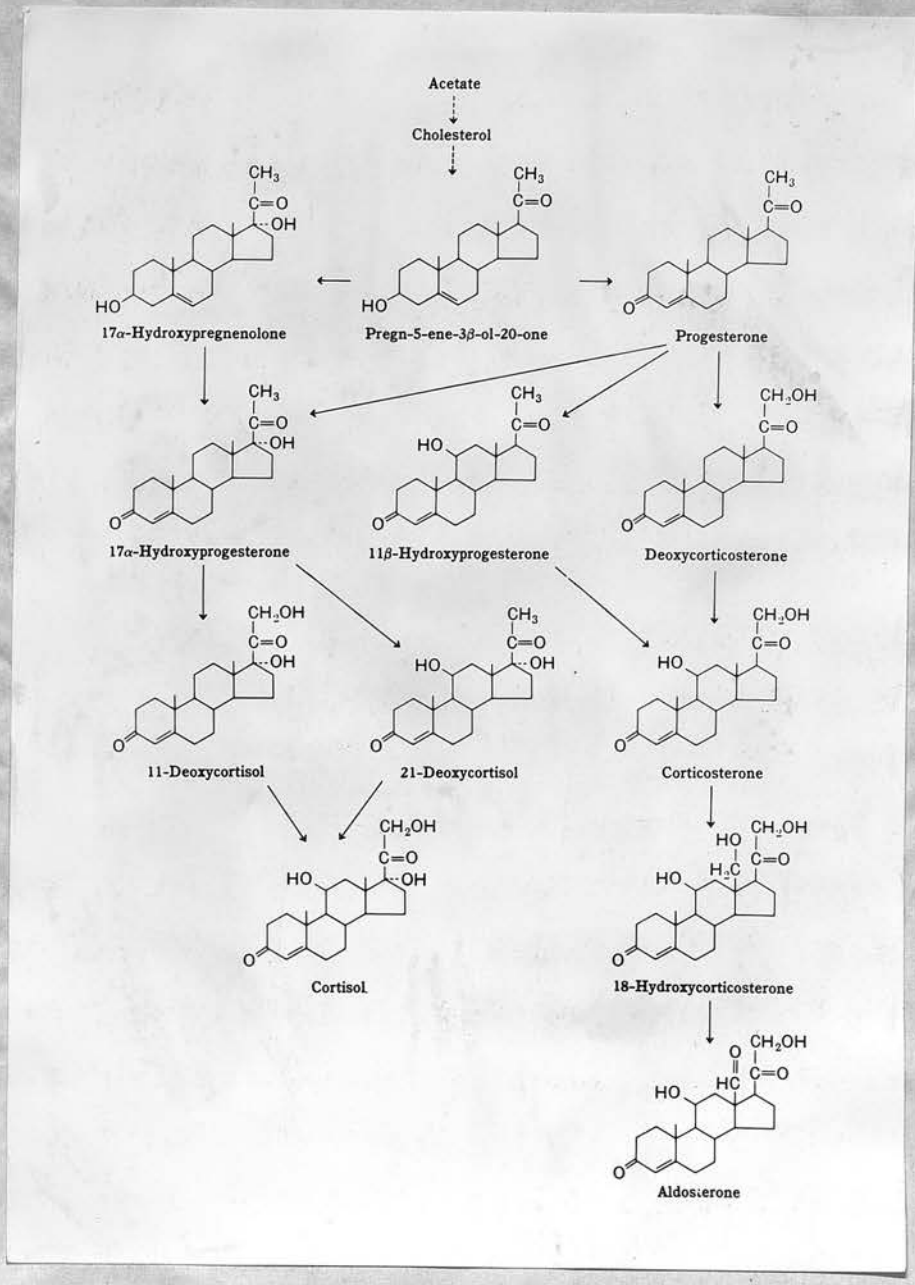


Fig.1. Pathways of formation of C_{21} steroids in adrenal cortex. (after "Principles of Biochemistry" McGraw-Hill, ed. White, Handler and Smith.)

would be expected to be the rate-limiting step in the respective biosynthetic pathway, and thus should be a key point in control on the one hand of bile acid formation by a feed-back mechanism involving the bile salts, and on the other of hormone production by the pituitary trophic hormones. Both reactions are similar, however, in that they appear to be mixed-function oxidase reactions (Mitton and Boyd, 1967; Halkerston, Eichhorn and Hechter, 1961).

Cholesterol Degradation in Endocrine Tissues

In each of the tissues synthesising steroid hormones therefore, the initial reaction is the removal of a six-carbon fragment from the side-chain of cholesterol to form a C_{21} steroid, pregnenolone. In adrenal cortex this is metabolised to a range of C_{21} steroids which are divided into two broad groups according to their physiological action, namely those affecting carbohydrate metabolism, and those affecting mineral metabolism. This division is somewhat arbitrary, as there is considerable overlap of these two ranges of activity. In man, the most important hormone of the former group is cortisol, and of the latter, aldosterone. Aldosterone biosynthesis is confined to the zona glomerulosa, (Simpson and Tait, 1955), while synthesis of the other hormones occurs in the zonae fasciculata and reticularis. A summary of the biosynthetic routes to the most important C_{21} steroids is shown in fig. 1. The order in which these reactions occur is not known and is probably somewhat variable. However, it is known that

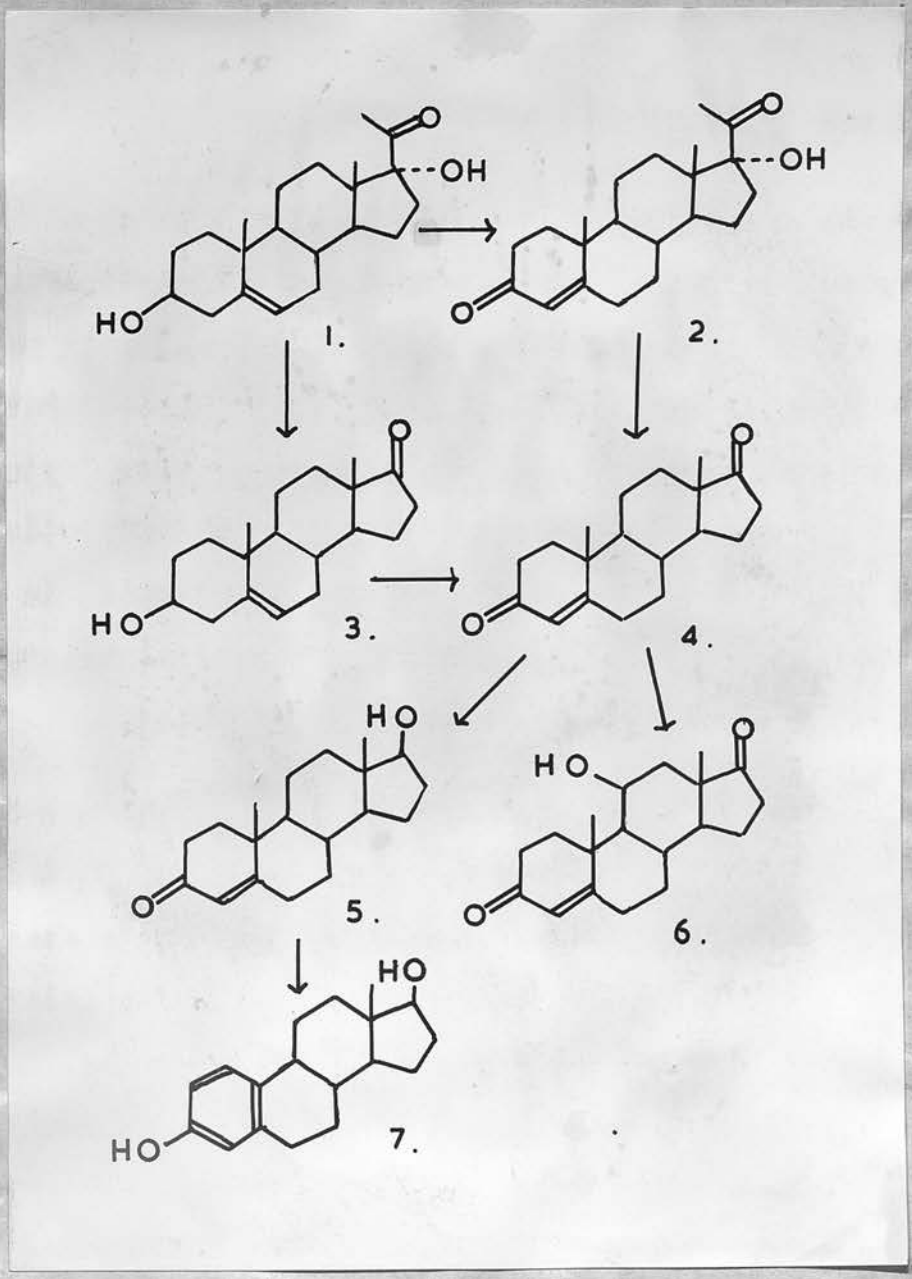


Fig.2. Pathways of formation of C_{19} and C_{18} steroids.

1. 17α-hydroxypregnenolone. 2. 17α-hydroxyprogesterone.
3. dehydroepiandrosterone. 4. androstenedione.
5. testosterone. 6. 11β-hydroxyandrostenedione.
7. oestradiol.

if hydroxylation at C_{21} occurs, hydroxylation at C_{17} cannot take place.

6β-hydroxylation of C_{21} steroids has been described for adrenal tissue of several species. 6β-hydroxycortisol is present in human urine. Hydroxylation at this position is particularly noticeable in cases of 11β-hydroxylation deficiency as in many adrenal carcinomas. The foetal adrenal possesses the ability to hydroxylate steroids at the 16α-position. 16α-hydroxylation of steroids is far less prominent in adults, reflecting a gradual alteration in the enzyme pattern with advancing gestation.

The androgens are C_{19} steroids formed from the C_{21} steroids, either 17α-hydroxyprogesterone or 17α-hydroxy-:pregnenolone, by removal of the two-carbon side chain. In the testes, androgen synthesis occurs in the interstitial cells. The most potent androgen is testosterone, but androstenedione and dehydroepiandrosterone also possess some androgenic activity. Some C_{19} steroids are also produced by the adrenal cortex, notably dehydroepiandro-:sterone, and this tissue is the chief source of 11-oxygenated C_{19} compounds such as 11β-hydroxyandrostenedione. This synthesis of androgens by the adrenal increases very markedly in congenital adrenal hyperplasia characterised by a deficiency in 11β- and 21-hydroxylating activities. A summary of the principal pathways of androgen formation is shown in fig. 2. In addition to these, a pathway to testosterone involving conversion of 17α-hydroxyprogesterone

to testosterone acetate has been proposed (Dorfman, 1962). Also, a pathway involving removal of the whole 8-carbon side-chain of cholesterol to yield the C_{19} steroids directly has not been definitely excluded.

The oestrogenic steroids are C_{18} compounds, as compared with the C_{19} androgens. In the naturally occurring oestrogens there is no angular methyl group at position 10, and ring A is aromatic. Although the ovaries and placenta are the chief sources of oestrogenic hormones in the human being, some are also produced by the adrenals and testes. The most potent oestrogen is oestradiol-17β (fig. 2). The immediate precursor of this is testosterone. The aromatisation enzyme system as studied in human placenta occurs in the endoplasmic reticulum and is a mixed-function oxidase, requiring NADPH and molecular oxygen. The 10-methyl group is oxidised either to formaldehyde or formic acid during the course of the reaction.

(3) Intracellular location of the steroid-metabolising enzymes in Endocrine Tissues

Cell fractionation studies have revealed that the cholesterol side-chain cleavage system of these tissues is located in mitochondria (e.g. Halkerston et al, 1961; Morrison, Meigs and Ryan, 1965; Sulimovici and Boyd, 1967). The 11β-hydroxylase and 18-hydroxylase of adrenal cortex are also mitochondrial enzymes (Sweat and Bryson, 1962). The 3β-ol dehydrogenase and Δ^5-3-ketosteroid isomerase enzymes are believed to be located in the endo-:plasmic reticulum, but these activities frequently occur in mitochondrial preparations (Morrison et al, 1965). This is of such a magnitude as to be unlikely to be due entirely to contamination of mitochondria with microsomes, so it has been suggested that mitochondria contain a 3β-ol dehydrogenase and Δ^5-3-ketosteroid isomerase as well as the endoplasmic reticulum (Koide and Torres, 1965). The aromatizing system of human placenta is also located in the endoplasmic reticulum, whereas the 17α-hydroxylating system of adrenal cortex appears to be localised in the particle-free supernatant (Young, Bryson and Sweat, 1965). Thus, there does not appear to be any common intracellular site of location of the steroid metabolising enzymes, and it must be assumed therefore that a cholesterol molecule in the course of its catabolism, to say cortisol, shuttles back and forth between mitochondria, endoplasmic reticulum and cytoplasm. It is likely that mitochondria are

frequently in intimate contact with the endoplasmic reticulum, so the distances traversed may be quite small, nevertheless the reason for this compartmentalisation is not known and its relation to overall steroid metabolism in the whole cell is not understood at present.

(4) The Precursor Pool of Cholesterol

There are many metabolic pools of cholesterol, both intercellular and intracellular, consequently the problem as to which cholesterol pool supplies the precursor of the steroid hormones synthesised by a particular tissue has demanded some attention. That this precursor pool of cholesterol might be only a small fraction of the total cholesterol within a tissue was first proposed by Hayano, Saba, Dorfman and Hechter (1956).

Armstrong, O'Brien and Greep (1964), working with rat ovarian slices, observed that the specific activity of progesterone biosynthesised from acetate-^{14}C was significantly higher than that of cholesterol in the same incubation and concluded that there was inhomogeneity of the ovarian cholesterol pool, with only a part of the pool being drawn upon for steroid biosynthesis. A similar conclusion was reached by Savard, Marsh and Rice (1965). Solod, Armstrong and Greep (1966) working with rabbit ovaries, further suggested that the cholesterol laid down most recently in the tissue whether derived from plasma cholesterol or newly synthesised *in situ*, may be the first to be drawn upon for pregnenolone formation. When the rate of steroid secretion is increased by luteinising hormone, cholesterol deposited earlier in the tissue is available for pregnenolone biosynthesis. That this latter cholesterol pool made available by the trophic hormone might be a cholesterol

ester fraction was suggested by Davis and Garren (1966), who showed that ACTH stimulated the conversion of cholesterol esters to free cholesterol in adrenals in vivo.

(5) The Physiological Substrate for Hormone Production

In recent years, the discovery was made that quantit-:atively the most important steroid produced by the adrenals was dehydroepiandrosterone and that this was secreted by these glands largely as the sulphate ester (Baulieu, 1962; Vande Wiele, MacDonald, Gurpide and Lieberman, 1963). In a healthy man, the secretion rate of dehydroepiandrosterone is 25-30 mg./day, whereas that of cortisol is some 10-20 mg./day and of aldosterone, only 250-500 µg./day. Subsequently, evidence has arisen to suggest that many steroids are metabolised as their sulphate esters. For example, in adrenal cortex, 17α-hydroxypregnenolone-3β-sulphate is metabolised directly to dehydroepiandrosterone sulphate in vitro (Lebeau, Alberga and Baulieu, 1964). Pregnenolone sulphate can be metabolised directly to 17α-hydroxypregnen-:olone sulphate by homogenates of hyperplastic adrenal tissue (Calvin and Lieberman, 1964). However, in their above-mentioned study, Lebeau and co-workers found that dehydroepiandrosterone sulphate was formed in higher yield from 17α-hydroxypregnenolone than from its sulphate, suggesting that removal of the side-chain followed by sulphatation is the preferred route.

Cholesterol sulphate is known to occur in adrenal cortex (Drayer, Roberts, Bandi and Lieberman, 1964), therefore it was necessary to investigate whether or not a pathway of side-chain cleavage of cholesterol sulphate to pregnenolone

sulphate was present in adrenal tissue. The existence of such a pathway was shown by Roberts, Bandi, Calvin, Drucker and Lieberman (1964) who demonstrated the *in vivo* metabolism of cholesterol sulphate to dehydroepiandrosterone sulphate without removal of sulphate from the molecule. However, Raggatt and Whitehouse (1966) compared the kinetics of side-chain cleavage of free cholesterol and cholesterol sulphate by adrenal cortex mitochondria and showed that the apparent Km for cholesterol sulphate was much greater than that for free cholesterol, indicating that free cholesterol is the preferred substrate for side-chain cleavage. Thus, although steroid sulphates are important end products of adrenal cortex metabolism, their role as precursors in this tissue appears to be a minor one.

In other tissues however, steroid sulphates may play an important role as precursors. For example, placental tissue can synthesise progesterone from cholesterol but is incapable of metabolising this further. The precursor of the C_{19} steroids required for oestrogen formation appears to be dehydroepiandrosterone and dehydroepiandrosterone sulphate synthesised either by the mother (Siiteri and MacDonald, 1963) or by the foetus (Bolté, Mancuso, Eriksson, Wiquist and Diczfalusy, 1964).

(6) Steroid Mixed-Function Oxidases

As has been seen, many of the reactions in steroid metabolism, such as cholesterol side-chain cleavage, aromatisation of androgens and hydroxylation reactions, involve insertion of molecular oxygen into the steroid molecule and require an electron donor. Mason (1957) has given the term "mixed-function oxidase" to such enzyme systems. The stoichiometry of mixed-function oxidase reactions of the hydroxylase type may be represented as:-

$$R - H + O_2 + XH_2 \longrightarrow R - OH + H_2O + X$$

where R - H is the substrate into which the molecular oxygen is being inserted, and XH_2 is the electron donor.

The incorporation of label from atmospheric oxygen ($O_2{}^{18}$) during the 11β-hydroxylation of deoxycorticosterone has been demonstrated by Hayano, Lindberg, Dorfman, Hancock and Doering (1955). Practically all mixed-function oxidases utilise one of the common cellular electron donors - the pyridine nucleotides NADH and NADPH. These are very powerful reducing agents - comparable with lithium aluminium hydride. Thus, we have the interesting situation of an oxidation reaction requiring a powerful reducing agent. This is the origin of the term "mixed-function oxidase", because both the substrate and the pyridine nucleotide are oxidised. Practically all steroid mixed-function oxidases

utilise NADPH as against NADH.

The nature of the activated form of oxygen which serves as the attacking agent is not known, but has received much attention, both from experimental and theoretical points of view. There is some evidence to suggest that the attacking species is electrophilic. Thus, HO^+ has been cited as a candidate, for example, in the cyclisation of squalene. Other possibilities suggested are the hydroxyl and perhydroxyl radicals.

New evidence as to the nature of the attacking oxygen species has come from the work of Staudinger (1966) who has compared the stereochemical nature of the products of non-enzymic model reactions which generated either hydroxyl radicals or oxygen atoms with that resulting from enzymatic hydroxylation of the same substrates. In each case, model systems generating oxygen atoms, but not those generating hydroxyl radicals, yielded hydroxylated products qualitatively similar to those of the enzymatic system. Thus, it seems that hydroxylation may be mediated by the generation of oxygen atoms rather than free hydroxyl radicals. The oxygen atom ($\cdot\underline{O}\cdot$) is a very powerful oxidant, capable of cleaving C - H bonds directly. Such a concept is very attractive in view of the likelihood of the involvement of cytochrome P-450 in mixed-function oxidation, as discussed in the next section. Staudinger suggests that the combination of cytochrome P-450 with oxygen is analogous

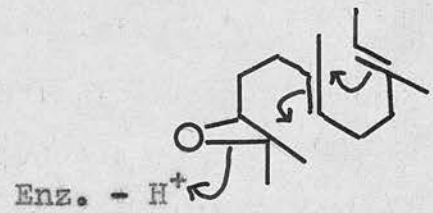
to the complex III of ferroperoxidase (Yamazaki and Yokota, 1965), which represents a stabilised form of the anion O_2^-. Reduction of this with the second electron of NADPH would lead to the active complex:-

$$Fe^{3+}_{cyt} \xrightarrow{+e} Fe^{2+}_{cyt} \xrightarrow{O_2} (Fe^{2+}_{cyt}\ O_2 \longleftrightarrow Fe^{3+}_{cyt}\ O_2^-)$$

"complex III"

$$\xrightarrow[-OH^-]{+e\ +\ H^+} (Fe^{3+}_{cyt} \cdot \underline{\underline{O}} \cdot \longleftrightarrow Fe^{4+}_{cyt}\ \underline{\underline{O}} \cdot \longleftrightarrow Fe^{5+}_{cyt}\ \underline{\underline{O}}^-)$$

Such a mechanism would be expected to lead to epoxide formation under suitable conditions. Recently, the possibility that epoxides may be involved as intermediates in some mixed-function oxidase reactions has been indicated with the demonstration that 2,3-oxidosqualene is a better precursor of sterols than is squalene, and also it is formed from squalene in a rat liver system. This would give the following mechanism for the cyclisation of squalene.



This mechanism does not involve the unlikely species HO^+ (Corey, Russey and Ortiz de Montellano, 1966). Whatever the details of the mechanism of oxygen attack however, it is obvious that the mixed-function oxidase must contain

enzyme sites for activation of oxygen, or substrate, or both of these. The system must also contain an enzyme site for transferring electrons from the pyridine nucleo-:tide to the reaction centre of the specific oxygenase. Hence, a mixed-function oxidase is a complex system, and one would not be surprised to find that it consisted of several protein components. The total free energy released in the transfer of two electrons from a reduced pyridine nucleotide to oxygen is about fifty Kilocalories. This reaction is therefore strongly exergonic and can provide the driving force for the mixed-function oxidase reaction.

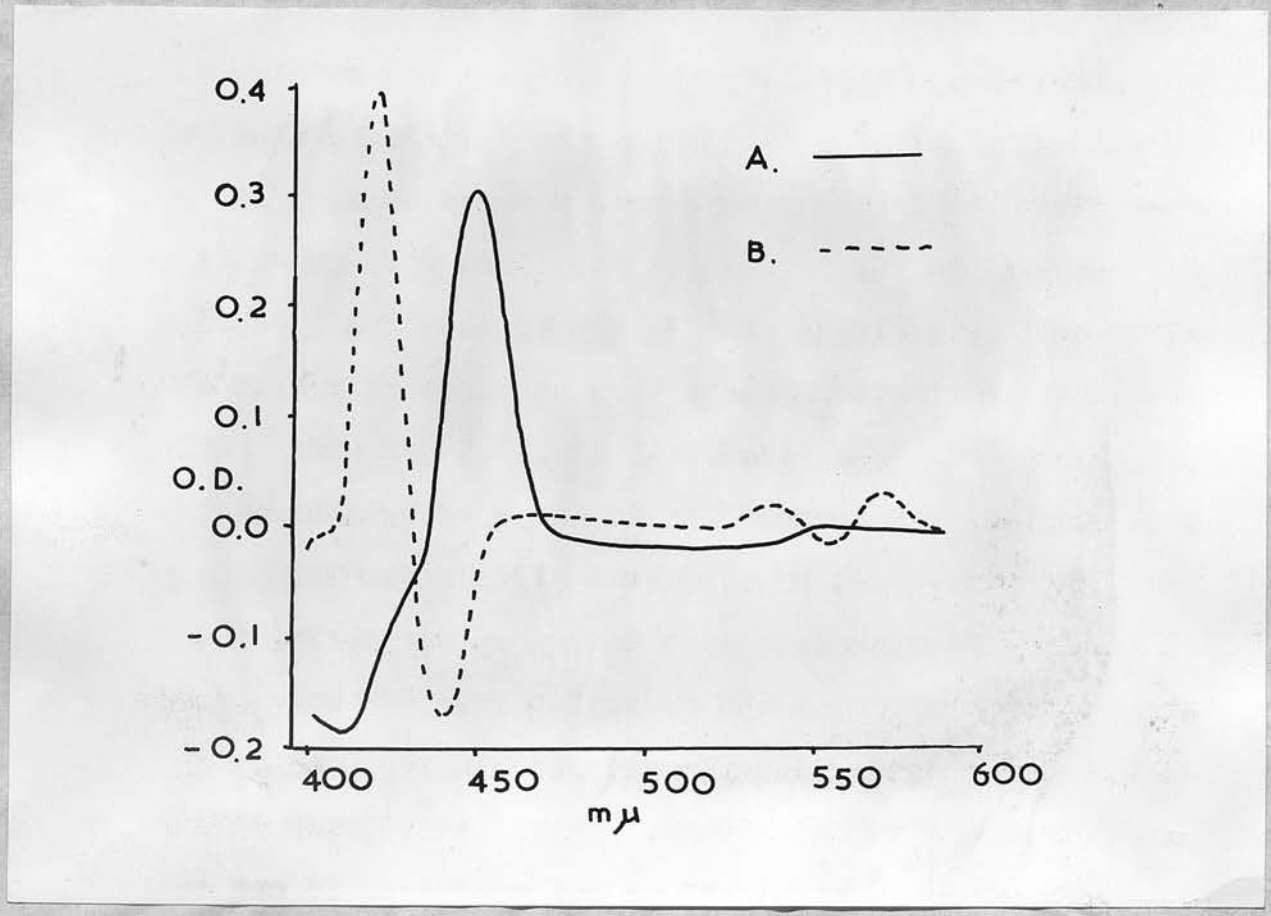


Fig. 3. Reduced carbon monoxide difference spectrum of rabbit liver microsomes. (after Omura and Sato, 1964a.)

A. "native" microsomes.

B. microsomes treated with deoxycholate or snake venom phospholipase.

(7) Cytochrome P-450

In 1958, Klingenberg and Garfinkel separately described in liver microsomes a pigment which, when reduced, combined with carbon monoxide to give an absorption maximum at 450 mμ. These observations were extended by Omura and Sato (1964a and b), who carried out a detailed chemical examination of the haem pigments of rabbit liver microsomes and found that only about 40 per cent of the haem content was accountable in terms of cytochrome b_5. When the microsomes were treated with snake venom phospho-:lipase or deoxycholate in the absence of oxygen, the absorption maximum at 450 mμ in the reduced carbon monoxide difference spectrum disappeared and was replaced by an absorption maximum at 420 mμ. When the pigment responsible for this chromophore at 420 mμ was purified, it was found to contain haem corresponding to the remaining 60 per cent of the haem content of the microsomes. This pigment could only have arisen by degradation of the original pigment with absorption maximum at 450 mμ, as cytochrome b_5 does not combine with carbon monoxide, and the microsomes were free from contaminating haemoglobin. These two forms of the pigment, native and degraded, were called 'P-450' and 'P-420', respectively. Their carbon monoxide reduced difference spectra are shown in fig. 3.

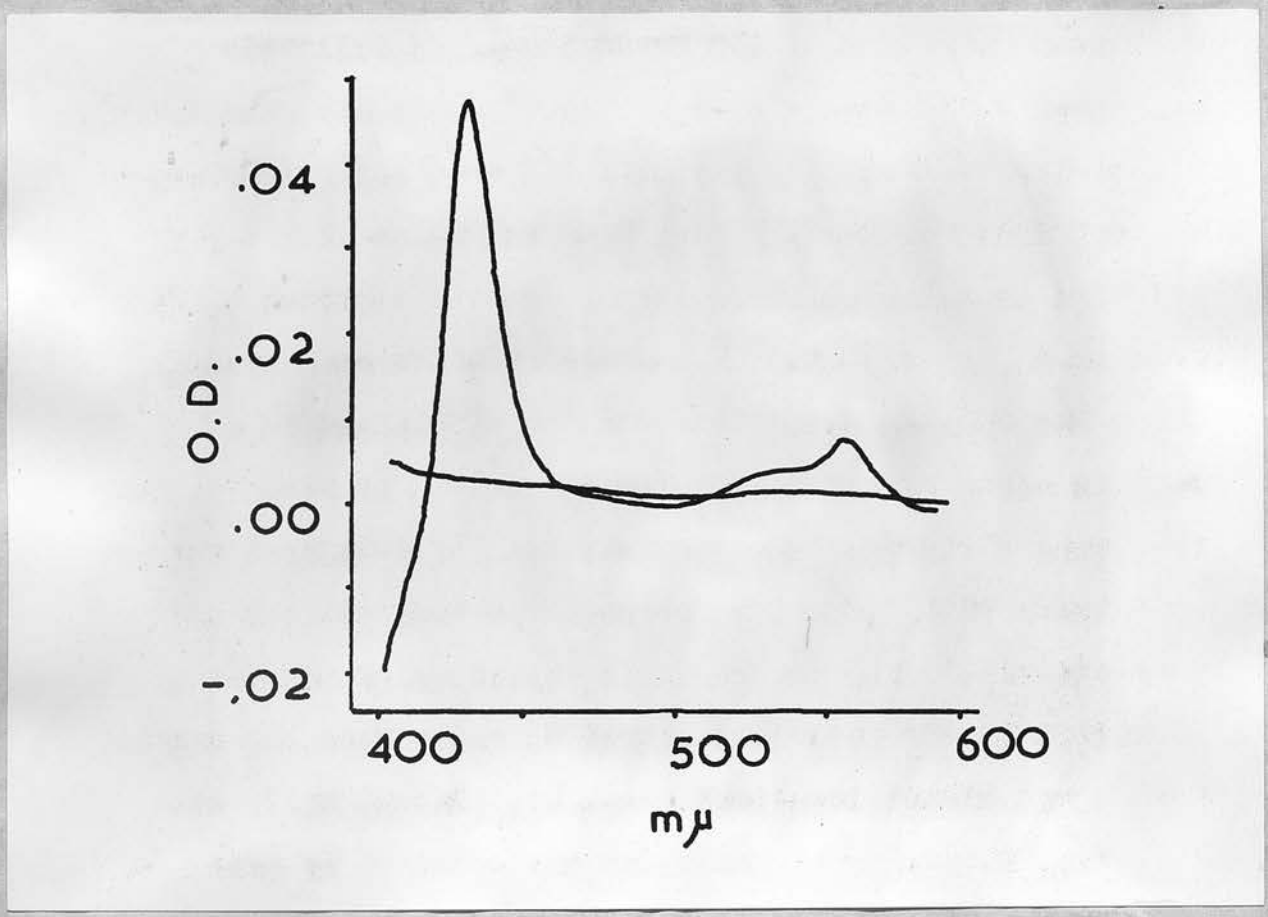


Fig.4. Difference spectrum of liver microsomes clarified with Lubrol W: anaerobic microsomes + NADH + NADPH minus aerobic microsomes + NADH.

When oxygen was admitted to the anaerobic cell, the chromophore disappeared as shown. (after Mason et al,1965.)

It was possible to obtain a reduced minus oxidised difference spectrum of the P-420 itself as follows:- When phospholipase-digested microsomes were kept anaerobic in the presence of dithionite, both P-420 and cytochrome b_5 were fully reduced. When NADH was added to the prep-:aration under aerobic conditions, only cytochrome b_5 was reduced to any extent. The spectral difference between these two samples would therefore be equivalent to a reduced minus oxidised difference spectrum of P-420. The resulting difference spectrum was that of a typical b-type cytochrome with α, β and Soret peaks at 500, 530 and 427 mμ respectively. The corresponding spectrum of microsomes clarified by the detergent Lubrol W, which does not degrade P-450, was almost identical (fig. 4), (Mason, North and Vanneste, 1965). This spectrum was obtained by substituting NADPH for dithionite, following the important observation that the 450 mμ absorption of the carbon monoxide complex of P-450 could be obtained by reduction with NADPH, although not with NADH. Thus, the reduced minus oxidised difference spectrum of P-450, and that of its degraded form, P-420, are both those of b-type cytochromes. The carbon monoxide reduced difference spectrum of P-420 is also typical of a haemoprotein. However, the carbon monoxide compound of P-450 is atypical, with its Soret band in the unusual position of 450 mμ, and apparently lacking in α and β-bands (fig. 3). Fig. 4 also illustrates the autoxidisability of P-450. In this respect it differs once again from

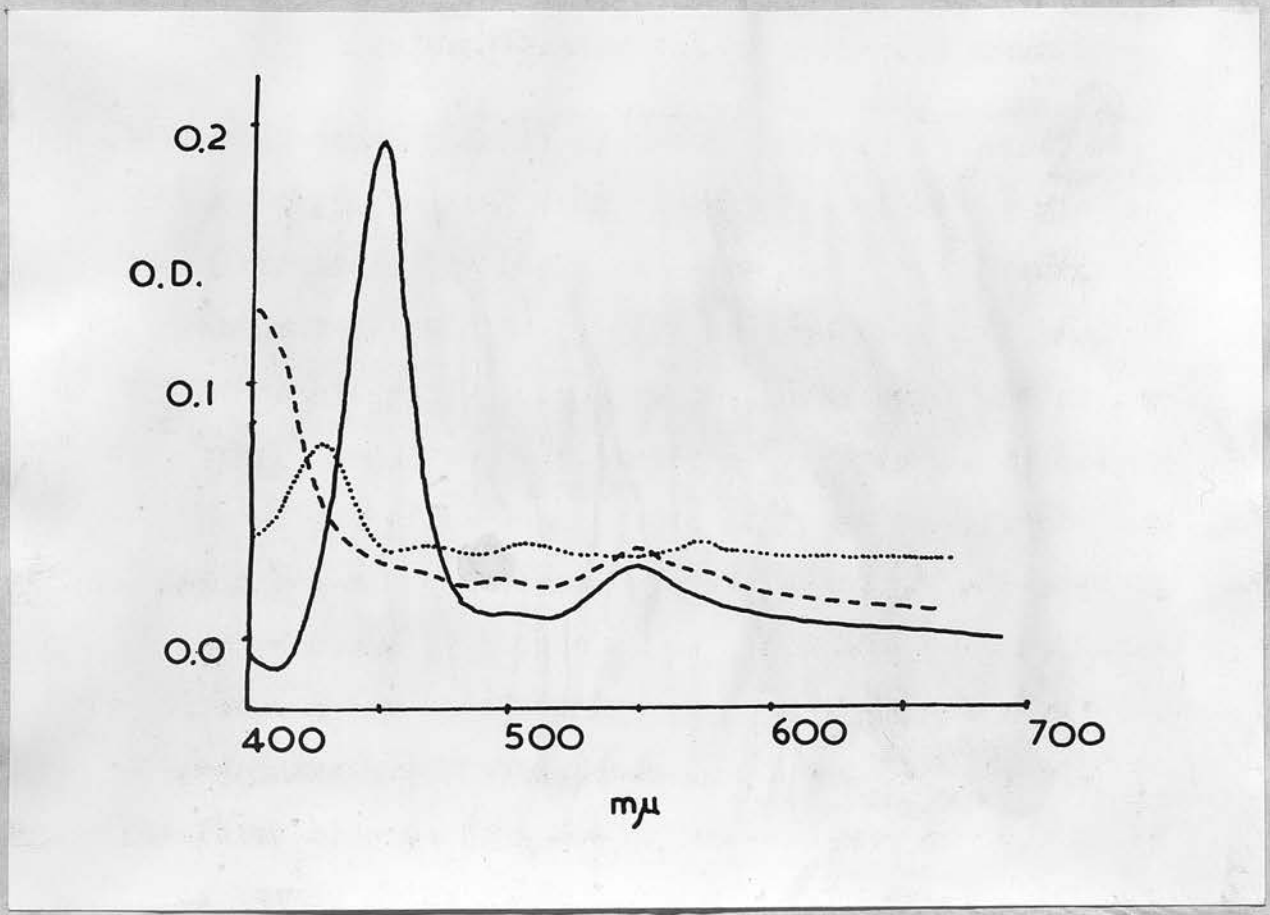


Fig.5. Absolute absorption spectrum of P-450 of adrenal cortex mitochondrial fragments.

Experimental cell - native preparation: reference cell - p-chloromercuribenzoate - treated preparation. Preparations in both cells were :-

..... in the oxidised state

– – – – reduced with dithionite.

——— reduced with dithionite and gassed with carbon monoxide.

(after Horie et al,1966.)

cytochrome b_5, which is not autoxidisable.

P-450 is known to occur in tissues other than liver, notably adrenal cortex microsomes (Estabrook, Cooper and Rosenthal, 1963) and adrenal cortex mitochondria (Harding, Wilson, Wong and Nelson, 1965). Sonicated fragments of the latter have been used by Horie, Kinoshita and Shimazono (1966) in an attempt to record the absolute, rather than the difference spectrum of P-450. Following the observation by Omura, Sato, Cooper, Rosenthal and Estabrook (1965) that P-450 could be converted to P-420 by yet another class of reagents, namely those which react with sulphydryl groups such as p-dichloromercuribenzoate, the above workers observed that prolonged aerobic treatment with this reagent gave a preparation which showed no absorption band whatever on treatment with carbon monoxide, and thus appeared to have selectively removed P-450 from the preparation. The difference spectrum of p-chloro-:mercuribenzoate treated preparation minus native preparation was therefore assumed to be the approximate absorption spectrum of P-450. This spectrum is shown in fig. 5. If this assumption is valid, it can be seen that the oxidised form had a low absorption peak at 427 mμ in the Soret region and a small peak in the visible region. The reduced form had a peak, which was higher than that of the oxidised form, around 400 mμ in the Soret region, and a broad peak at 547 mμ in the visible region. Other haemoproteins have the reduced Soret band at longer

wavelengths than the oxidised Soret band, and this makes a sharp contrast with fig. 5. When reduced and gassed with carbon monoxide, the spectrum showed the characteristic absorption at 447 mμ, and a broad peak at 554 mμ in the visible region, very similar to that of the reduced spectrum. This could account for the failure to observe α and β bands in difference spectra of P-450 (fig. 3). The carbon monoxide-shift of the Soret peak of usual haemoproteins occurs to shorter wavelengths, and this again contrasts sharply with fig. 5. Essentially the same spectra were obtained for P-450 from liver microsomes (Kinoshita and Horie, 1967).

Microsomal Fe_x

The work of Mason and co-workers (e.g. Mason et al, 1965) has shown that microsomes from liver and other tissues contain an oxidation-reduction component detectable by electron spin resonance spectroscopy (ESR). It displays a characteristic signal with $S_m = 2.41$, $g_m = 2.25$ and $S_m = 1.91$. This signal is similar to those of low-spin ferric haemoproteins (Morita and Mason, 1965) and so the component has been called "microsomal Fe_x". The quantity of microsomal Fe_x present in native liver microsomes estimated by integration of the ESR signal, was found to be equal to the quantity of P-450, estimated from the optical absorption. Either accounted for all the microsomal haem other than that of cytochrome b_5. In addition,

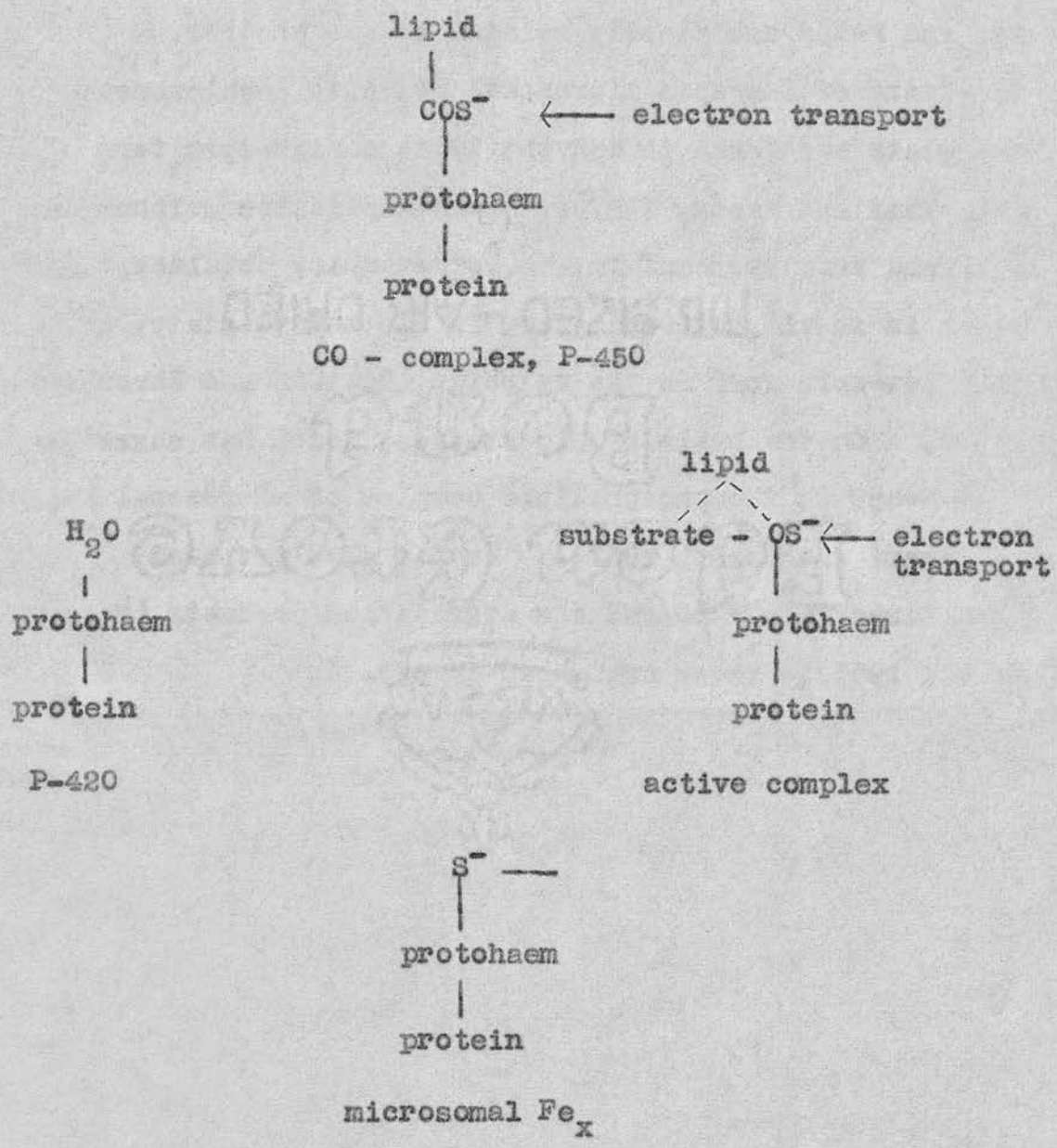


Fig.6. Proposed structures for P-450 and its derivatives. (after Mason et al.,Fed. Proc. 24, 1172 (1965).)

microsomal Fe_x, like P-450, was reduced by NADPH, and was readily autoxidisable. This suggests that microsomal Fe_x and P-450 are closely related to one another. Treatment of low-spin microsomal Fe_x with p-chloromercuri-:benzoate was found to convert it to a high-spin form (Murakami and Mason, 1967). This implicates sulphur as a ligand in microsomal Fe_x. For example, catalase, which is in the high-spin form in its native state, exists in a low-spin form as the sulphide (Deutsch and Ehrenberg, 1952). On the basis of his studies, Mason has suggested that P-450 is the phospholipid complex of microsomal Fe_x (Murakami and Mason, 1967). He has also proposed structures for P-450 and its degradation products (Mason et al, 1965); these are shown in fig. 6.

(8) The Effect of carbon monoxide on steroid mixed-function oxidases

In 1957, Ryan and Engel observed that the C-21 hydroxylation of 17-hydroxyprogesterone by adrenal cortex microsomes was inhibited by carbon monoxide. Following their observation that P-450 occurred in adrenal cortex microsomes, Estabrook et al (1963) carried out experiments to see if this carbon monoxide-binding pigment had any relation to the observed carbon monoxide inhibition of this mixed-function oxidase reaction. They observed that the equilibrium constant for the displacement of oxygen by carbon monoxide in both cytochrome P-450 and the steroid 21-hydroxylase was equal to about unity, which contrasts markedly with the values for other carbon monoxide-binding haemoproteins such as cytochrome oxidase and haemoglobin which are 10 and 1.8 - 8.0 x 10^{-3} respectively (Keilin and Wang, 1946). This suggested the oxygen-binding site for the steroid 21-hydroxylase might indeed be cytochrome P-450. However, this is not in itself proof of such a relationship as the similarity might be fortuitous. A more conclusive test would be to compare the absorption spectrum of the carbon monoxide-inhibited form of the steroid 21-hydroxylase with that of cytochrome P-450 to see if they were identical.

A method for establishing the absorption spectrum of the carbon monoxide complex of the terminal oxidase of the respiratory chain was established by Warburg (1949) who shone monochromatic light of different wavelengths through

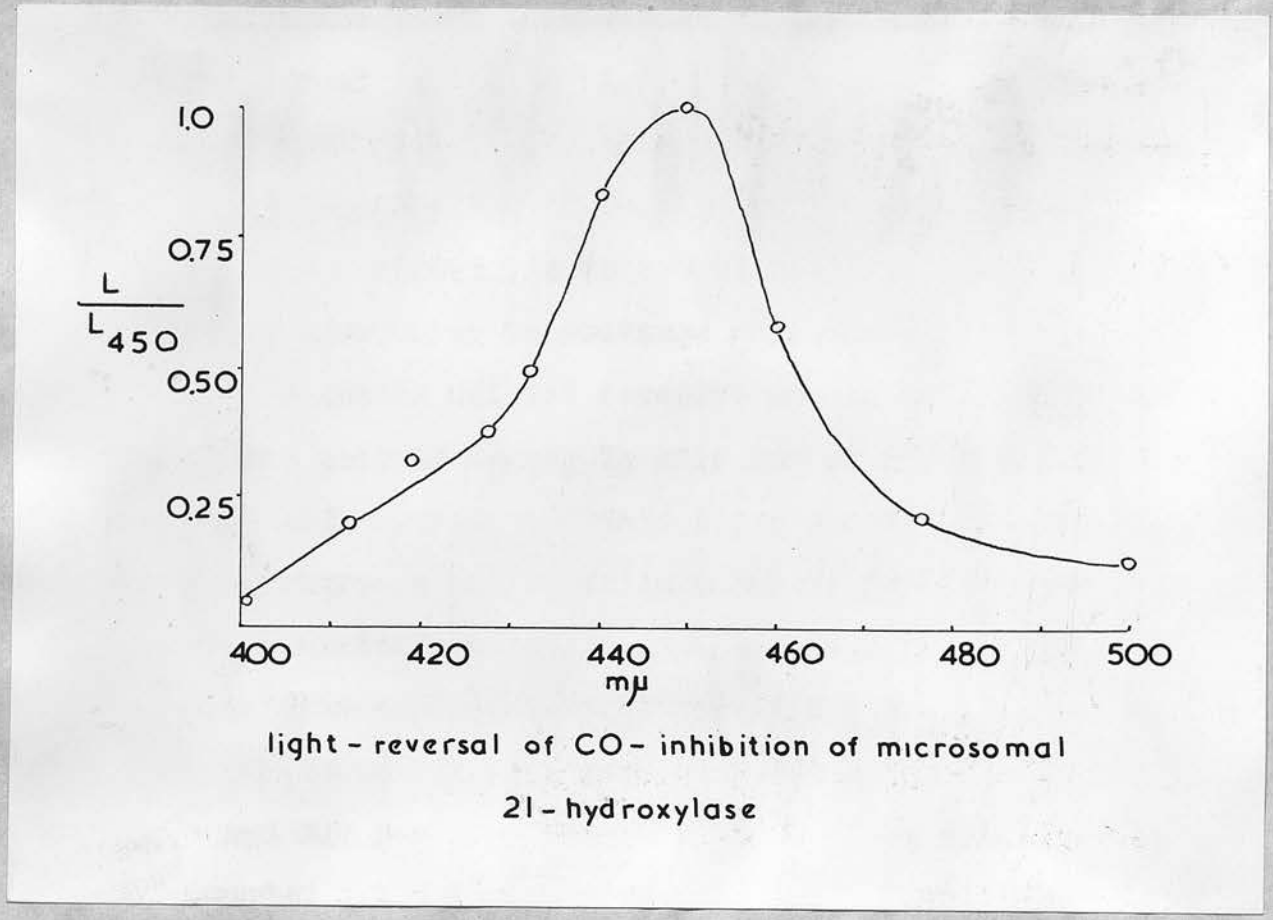


Fig.7. Photochemical action spectrum for light-reversal of the carbon monoxide inhibition of the steroid 21-hydroxylase. (after Omura et al,1965.)

the preparation and plotted degree of reversal of carbon monoxide-binding against wavelength. The resulting photochemical action spectrum is identical to the optical absorption spectrum of the material. When this was done for the steroid 21-hydroxylase, the action spectrum shown on fig. 7 was obtained (Omura et al, 1965). Comparison of this with the absorption spectrum of cytochrome P-450 on fig. 3 provides strong evidence for the identity of cytochrome P-450 as the site of oxygen binding and activation for the steroid 21-hydroxylase. These workers also demonstrated that a similar action spectrum could be observed for several drug-metabolising mixed-function oxidase reactions of liver microsomes:- the oxidative demethylation of codeine to morphine, the oxidative demethylation of monomethylaminopyrine, and the hydroxylation of acetanilide. These are systems which are induced on administration of the appropriate drug. It has been observed that as the mixed-function oxidase activity increases on such administration, there is a corresponding rise in the P-450 content of the liver microsomes (Orrenius, Dallner and Ernster, 1964).

The steroid 11β-hydroxylase of adrenal cortex mitochondria has also been shown to be inhibited by carbon monoxide (Wilson, Nelson and Harding, 1965), and the photochemical action spectrum for light-reversal of this inhibition is similar to that of the 21-hydroxylase (Cooper, Novack, Foroff, Slade, Sanders, Narasimhulu and Rosenthal, 1967).

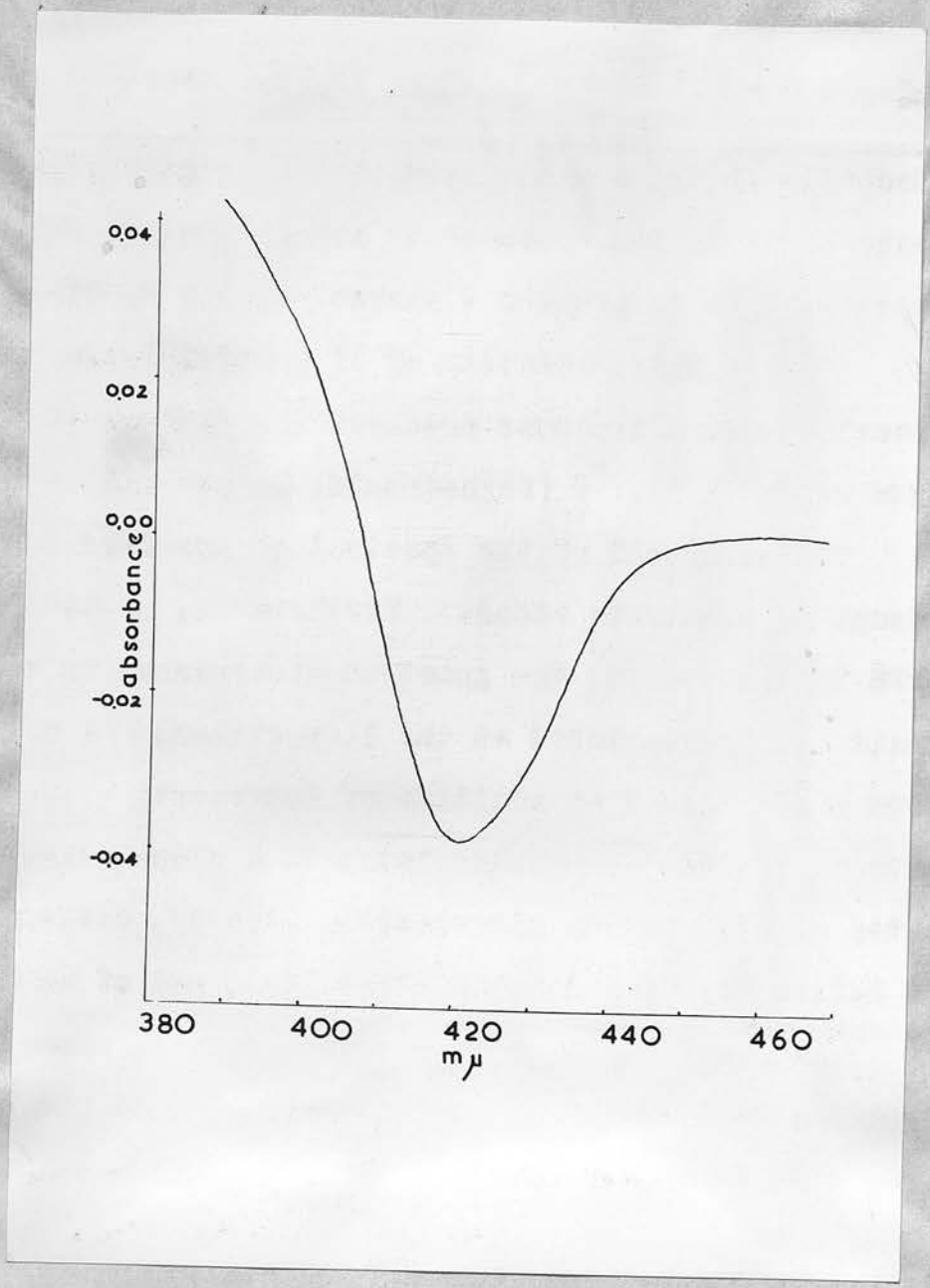


Fig.8. Difference spectrum induced by treatment of adrenal cortex microsomes with 17α-hydroxyprogesterone. Reference cell - microsomes; experimental cell - microsomes plus steroid. (after Narasimhulu et al,1965.)

Thus, P-450 appears to be involved in this reaction also.

Recently evidence has accumulated to suggest that a substrate added to its appropriate mixed-function oxidase interacts with it to produce a characteristic spectral change. For example, addition of 17α-hydroxyprogesterone to adrenal cortex microsomes produced the difference spectrum shown in fig. 8 (Narasimhulu, Cooper and Rosenthal, 1965). The magnitude of the spectral change depended on the amount of substrate added. Furthermore, on addition of NADPH to the system, the spectrum disappeared as the substrate was hydroxylated at the 21-position. A similar spectrum was obtained on addition of deoxycorticosterone, a substrate for the 11β-hydroxylase, to a preparation of sonicated adrenal cortex mitochondria (Cooper, Narasimhulu, Slade, Raich, Foroff and Rosenthal, 1965), and of aminopyrine and hexobarbital to liver microsomes. In some cases, the relationship between the substrate concentration required to cause a half-maximal spectral change has been compared with the affinity of the liver mixed-function oxidase for substrate hydroxylation. For example, in the case of aminopyrine (which is hydroxylated to form formaldehyde and aminoantipyrine) there was a similarity between the enzymatically determed K_m and that determined optically (Remmer, Schenkman, Estabrook, Sasame, Gillette, Narasimhulu, Cooper and Rosenthal, 1966).

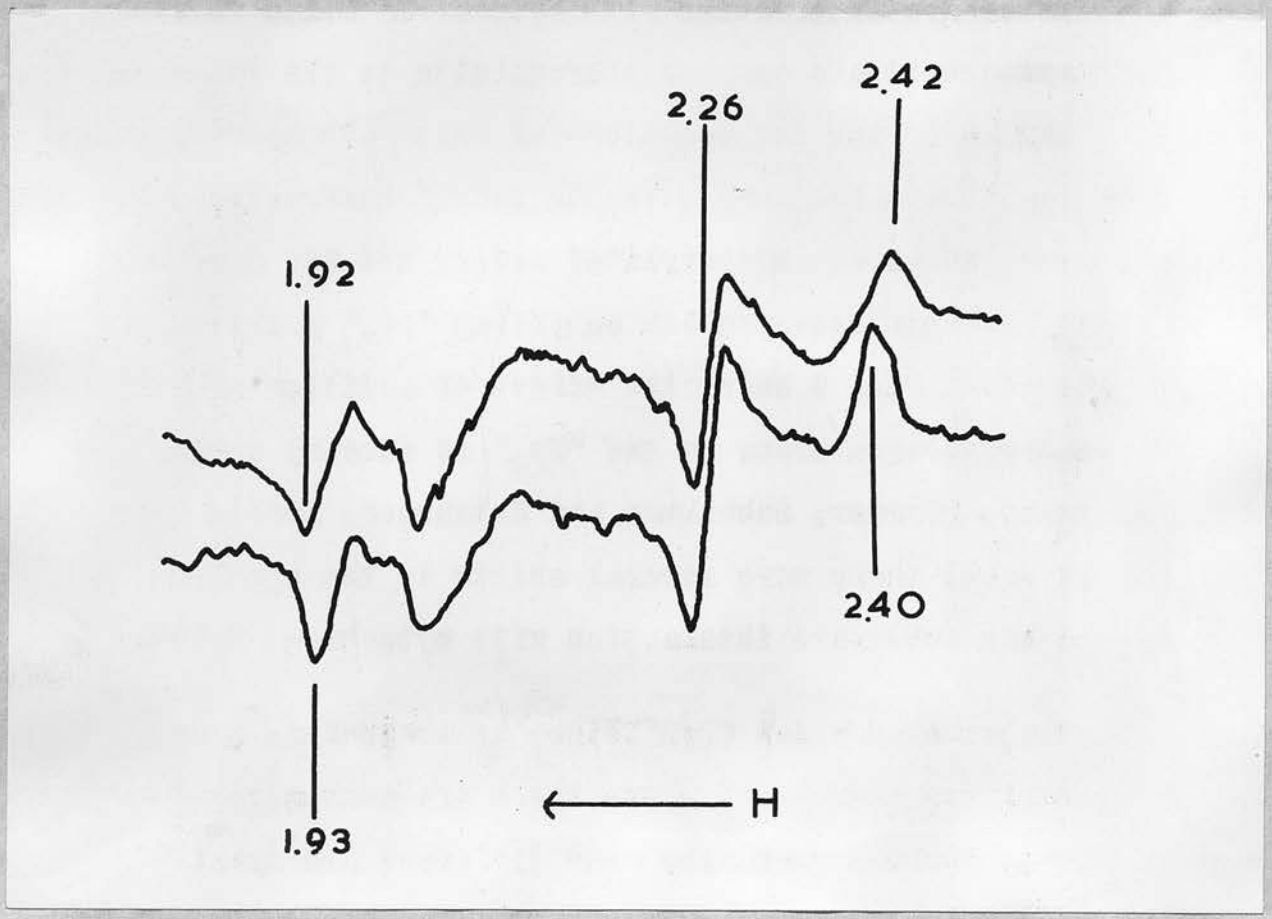


Fig.9. ESR spectra (first derivative) of adrenal cortex microsomes at the temperature of liquid nitrogen.

lower spectrum - untreated.

upper spectrum - in the presence of 17α-hydroxyprogesterone.

(after Cammer et al,1966.)

These observations have led to the hypothesis that substrates are interacting with cytochrome P-450 forming an enzyme-substrate complex prerequisite to the interaction with oxygen during the reactions of substrate hydroxylation. This hypothesis has been strengthened by observations that compounds which are hydroxylated modify the ESR spectrum of the low-spin haemoprotein component "Fe_x" previously discussed. Fig. 9 shows the effect of addition of 17α-hydroxyprogesterone on the "Fe_x" of adrenal cortex microsomes (Cammer, Schenkman and Estabrook, 1966). As can be seen, there were several shifts in the spectrum, suggesting substrate interaction with cytochrome P-450.

The problem which then arises is whether in a case such as liver microsomes where there are many mixed-function oxidases, there are equally many different and specific forms of cytochrome P-450, or whether the different systems all utilise a common pool of P-450, and specificity lies in some other component. The evidence at present suggests at least two forms of P-450 in liver microsomes, because whereas hexobarbital and aminopyrine produce the spectral shift shown in fig. 8 on interaction with the mixed-function oxidase system, substrates such as aniline, pyridine and nicotinamide produce a different kind of spectral shift with a maximum at 430 mμ and a trough at 393 mμ. However, much more work is required to throw more light on this problem.

Although there is strong evidence for the involvement of cytochrome P-450 in many mixed-function oxidase reactions, it does not appear to be involved in all. For example, no evidence has been presented for its involvement in phenylalanine hydroxylase, a pteridine-requiring hydroxylase (Kaufman and Levinberg, 1959), nor in similar systems such as tyrosine hydroxylase. Neither does it appear to be involved in the desaturation of fatty acids (Oshino, Imai and Sato, 1966). Perhaps the situation in mixed-function oxidation is similar to that in utilisation of oxygen generally, where although in the mitochondrial respiratory chain electrons pass from the pyridine nucleotides via flavoproteins to several cytochrome components before reducing the oxygen, there are many enzymes which reduce oxygen by means of electrons from a pyridine nucleotide but do not possess cytochromes, the electron transport chain stopping short at a flavin.

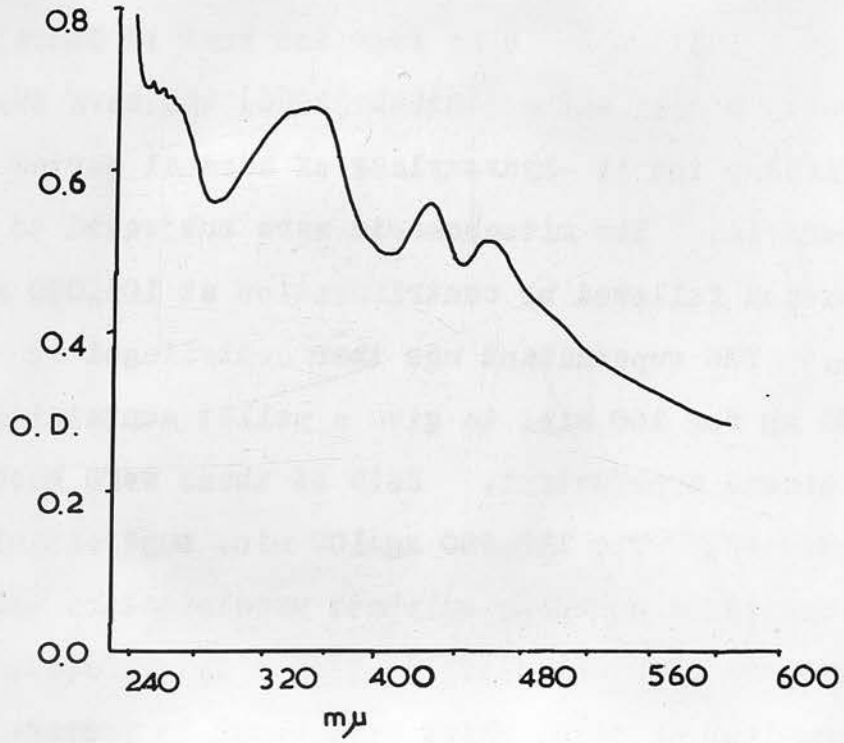


Fig.10. Absorption spectrum of adrenodoxin in 0.01M phosphate buffer pH 7.4 . (after Kimura and Suzuki, 1967.)

(9) Purification of the steroid 11β-hydroxylase

Further insight into the mechanism of steroid mixed-function oxidation has come from the work of Omura, Sanders, Estabrook, Cooper and Rosenthal (1966) who have succeeded in purifying the 11β-hydroxylase of adrenal cortex mitochondria. The mitochondria were subjected to ultra-:sonication followed by centrifugation at 105,000 xg for 30 min. The supernatant was then centrifuged at 150,000 xg for 100 min. to give a pellet containing P-450 and a second supernatant. Both of these were necessary for activity. The 150,000 xg-100 min. supernatant was fractionated by ammonium sulphate precipitation and chroma-:tography on DEAE-cellulose to give a flavoprotein and a non-haem iron protein, which were shown to possess NADPH-cytochrome P-450 reductase activity. Combination of all three fractions reconstituted the 11β-hydroxylase activity. This non-haem iron protein has been termed "adrenodoxin" and has a molecular weight of about 20,000. Each molecule contains two atoms of iron and two atoms of acid-labile sulphur (Kimura and Suzuki, 1967). The absorption spectrum is shown on fig. 10 and is remarkably similar to that of spinach ferredoxin, as is the ESR spectrum, with a signal at $g_{\perp} = 1.94$ in the reduced form. However, it differs from ferredoxin in that the oxidation-reduction potential is high, E_o' at pH 7.4 = 0.164 volts. Thus, the 11β-hydroxylase is an electron transport system analogous to the mitochondrial respiratory chain, except that in

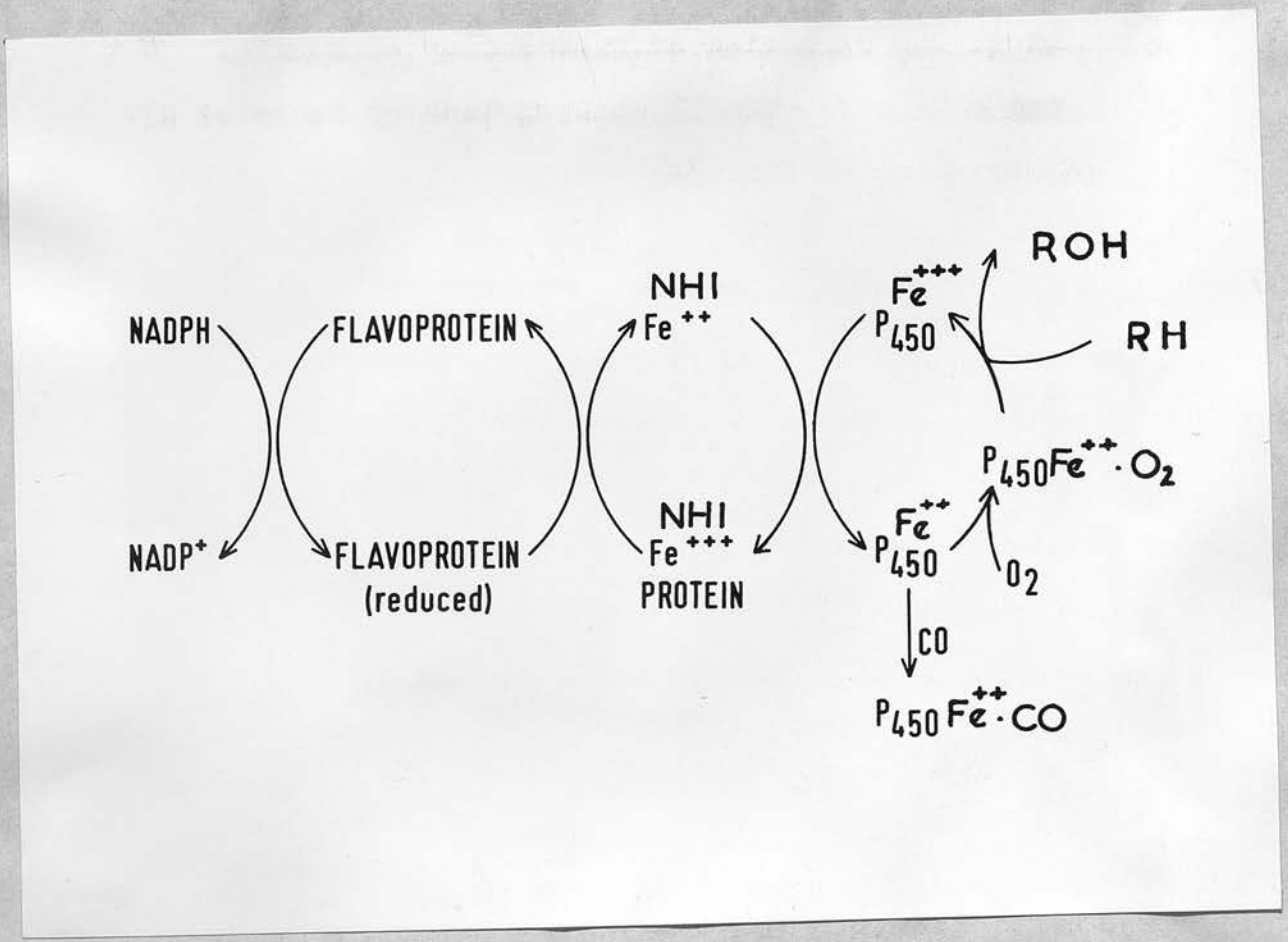


Fig. 11. Proposed sequence for electron transport in the 11β-hydroxylase. (after Omura et al,1966.)

the former, electron transport does not appear to be coupled to the formation of high-energy phosphate. The proposed scheme for the 11β-hydroxylase of Omura et al (1966) is shown in fig. 11.

(10) The cholesterol side-chain cleavage system

This enzyme system occurs in the mitochondria of adrenal cortex, placenta, ovaries and testes. As mentioned previously, the cleavage of the cholesterol side-chain is the first reaction in the catabolism of cholesterol to the steroid hormones and thus would be expected to be a key reaction in the control of hormone formation. Yet in spite of its obvious importance, little is known of the mechanism of the cleavage reaction, nor of the enzymes involved. This is partly due to the difficulty of assay. Most methods have relied on radioisotope techniques for detection, and paper chromatography of the steroid products formed (e.g. Halkerston et al, 1961), or steam distillation to separate the 6-carbon side-chain fragment (Constantopoulos and Tchen, 1961a). These methods are time-consuming and tedious. Roberts, Creange and Young (1965) used thin-layer chromatography for separation of the steroid products, but the resolving power of their solvent systems, utilising benzene and ethyl acetate, was poor. Thus, the study of this reaction would be greatly facilitated by the develop-:ment of a simple and rapid assay.

It is known, however, that the reaction requires NADPH and molecular oxygen (Halkerston et al, 1961) and thus partially fulfils the requirements for a mixed-function oxidase. It has been suggested that the cleavage of the side-chain occurs via a series of hydroxylation

reactions as follows:-

cholesterol ⟶ 20α-hydroxycholesterol

⟶ 20α,22ξ-dihydroxycholesterol⟶ pregnenolone

Evidence for these hydroxylated intermediates is based chiefly on incubation studies in which these compounds have been shown to be metabolised to pregnenolone more readily than the parent cholesterol (Shimizu, Hayano, Gut and Dorfman, 1961; Shimizu, Gut and Dorfman, 1962; Constantopoulos and Tchen, 1961b). Hall and Koritz (1964) working with acetone powders of bovine adrenal cortex mitochondria have shown that 20α-hydroxycholesterol non-competitively inhibits the side-chain cleavage of cholesterol, suggesting that the 20α-hydroxycholesterol may be occupying a binding site other than that occupied by cholesterol. This could be the site occupied by 20α-hydroxycholesterol formed from cholesterol in the above reaction sequence. However, attempts to detect these compounds when isotope-labelled cholesterol is the substrate have led to conflicting results. Hall and Koritz in their above-mentioned study were unable to find any radioactivity in the re-isolated 20α-hydroxycholesterol. These workers also found no accumulation of 20α-hydroxycholesterol when the system was inhibited with pregnenolone (Koritz and Hall, 1964). On the other hand, Solomon, Levitan and Lieberman (1956) apparently did obtain isotope-labelled "20-hydroxycholesterol" when non-labelled material was added as a trapping agent.

Ichii et al (1963) found both 20α-hydroxycholesterol and 20,22-dihydroxycholesterol when the reaction was inhibited by addition of pregnenolone, using acetone powders of bovine corpora lutea. Finally, Constantopoulos, Satoh and Tchen (1962), using preparations from bovine adrenal cortex, found 20,22-dihydroxycholesterol accumulated when large amounts of pregnenolone and progesterone were added to the incubation medium. Thus, the evidence is conflicting and considerable doubt remains as to the nature of the intermediates between cholesterol and pregnenolone.

The nature of the 6-carbon fragment split from the cholesterol molecule is better established. This is generally isolated from incubations as isohexanoic acid (Shimizu, Dorfman and Gut, 1960). However, if unlabelled isohexanoic aldehyde was added to an incubation with cholesterol -26-C^{14} as substrate, labelled isohexanoic aldehyde accumulated (Constantopoulos and Tchen, 1961a). This suggests the aldehyde is formed first and is rapidly dehydrogenated to the corresponding acid.

Fractionation of the cholesterol side-chain cleavage system

Following reports that the cholesterol side-chain cleavage enzyme system could be extracted from acetone powders of adrenal cortex mitochondria with phosphate buffer (e.g. Halkerston et al, 1961), attempts were made

to fractionate this extract using ammonium sulphate. Bryson and Kaiser (1962) and Constantopoulos and Tchen (1961a) found that the extract could be resolved into two protein fractions by this treatment, both of which were required to reconstitute side-chain cleavage activity.

This was virtually the total knowledge of the chemistry and enzymology of the cholesterol side-chain cleavage system at the time of commencement of the work to be reported here.

(11) Aims of the Present Study

A study of the side-chain cleavage of cholesterol by adrenal cortex was undertaken because little was known about this reaction, yet it is one of the key reactions in the synthesis of steroid hormones from cholesterol, being the first step in this metabolism.

It was necessary to develop a rapid and simple assay of the side-chain cleavage reaction. When this was achieved, the mechanism of the reaction was studied and the protein components of the enzyme system effecting this cleavage reaction were investigated. This latter study involved a detailed investigation of the effects of carbon monoxide and light on the system and also fractionation and purification of the protein components involved. Study of this reaction was undertaken from a strictly mechanistic point of view, firstly because this presented a fascinating problem in itself, and secondly, because it was felt that any fundamental knowledge of the control of the system must presuppose a detailed description of the system itself.

2. EXPERIMENTAL PROCEDURE

(1) Introduction

The object of this work, as discussed in the General Introduction, was to study the cholesterol side-chain cleavage system of adrenal cortex. A plentiful source of enzyme was required and for this reason the tissue of choice was bovine adrenal cortex, as the adrenal glands of cattle are large and a plentiful supply was available from the Edinburgh Corporation Abbatoir. The first part of this chapter describes the method of tissue preparation, isolation of sub-cellular fractions, and the methods used to solubilise the cholesterol side-chain cleavage enzyme system. The second part describes the developments of a rapid and extremely sensitive assay method for this reaction.

Materials and chemicals used throughout the work are listed in Appendix 1. Instruments used are listed in Appendix 2. Analytical techniques other than those mentioned in the text are described in Appendix 3.

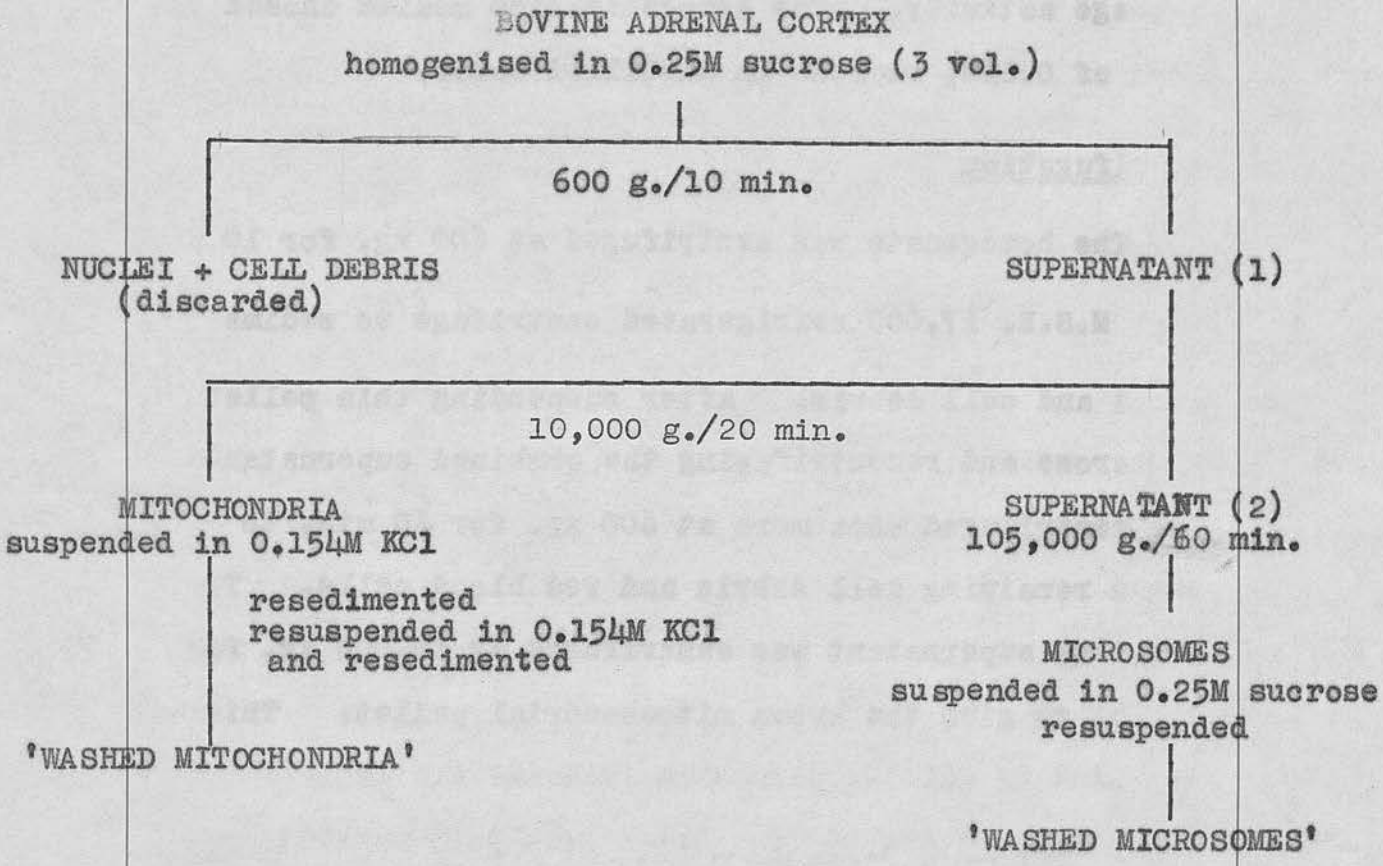
(2) Tissue Preparation

The glands were removed from the animals, generally bullocks or heifers, usually between 10-20 min. after death, and transferred immediately to crushed ice. Generally it was found convenient to collect 8-14 glands at a time. The glands were transported to the laboratory in about 30 min.

Homogenisation

All subsequent operations were performed at 0-4°C. The glands were trimmed free of surrounding fat and slit down the middle to reveal the pale brown medulla and the surrounding cortex, which was a deep red colour. As much of the medullary tissue as was practicable was removed, leaving the cortex plus capsule, which together usually weighed 7-8 g. As it proved extremely difficult to remove the capsule from the cortex, attempts were made to homogenise with the capsule still attached. This could not be achieved with a homogeniser consisting of a Teflon pestle and glass tube, but an all-glass homogeniser was satisfactory, providing the tissue was first chopped finely with scissors. The pestle was quite loose-fitting and had welded glass knobs at the bottom. All the surfaces in contact with the tissue were ground. The pestle was connected to the drive motor by a short length of rubber pressure tubing. When preparing large batches, 10 glands or more, the tissue was homogenised for 30 sec. at half-speed in a Waring Blendor

Table 1



Centrifugal Fractionation of Homogenate

prior to transfer to the all-glass homogeniser. This did not result in any noticeable loss of cholesterol side-chain cleavage activity. The homogenisation medium chosen was 3 v/w of 0.25M. sucrose in distilled water.

Centrifugation

The homogenate was centrifuged at 600 xg. for 10 min. in an M.S.E. 17,000 refrigerated centrifuge to sediment nuclei and cell debris. After suspending this pellet in sucrose and recentrifuging the combined supernatants were centrifuged once more at 600 xg. for 10 min. to remove remaining cell debris and red blood cells. The resultant supernatant was centrifuged at 10,000 xg. for 20 min. to give the brown mitochondrial pellet. This was suspended in 0.154M. potassium chloride and centrifuged for 10 min. at 10,000 xg. Repeating this washing gave the washed mitochondrial pellet.

The supernatant obtained after initially sedimenting the mitochondria was centrifuged at 105,000 xg. for 60 min. in a Spinco Model E ultracentrifuge to sediment the dark red microsomes. The resultant supernatant was a clear reddish colour. The microsomes were generally resuspended in 0.25M. sucrose and resedimented. This procedure is summarised in Table I.

Ultrasonication of mitochondria

The twice-washed mitochondrial pellet was suspended by hand homogenisation in two volumes of distilled water and subjected to ultrasonication at 0°C for 15 min. with intervals for cooling every 5 min., using an M.S.E.-Mullard ultrasonic disintegrator operating at 20Kc./sec. (Cooper et al, 1965). The sonicate could be stored at -15°C for several weeks without loss of activity. Twelve glands yielded roughly 50 ml. sonicate.

Preparation of acetone powder of mitochondria

The twice-washed mitochondrial pellet was suspended by hand homogenisation in two volumes of 0.154M. potassium chloride. The mitochondrial suspension was poured into 15 volumes of acetone and cooled in an acetone-solid carbon dioxide bath. The suspension was poured into the acetone drop by drop while the acetone was violently agitated. This ensured immediate dispersion of the water in each drop and prevented it freezing round the protein. The suspension was then filtered at a water pump in a Buchner funnel and washed five times with acetone cooled in the acetone-carbon dioxide bath. The protein pellet was not allowed to dry out during this process, and was kept continuously stirred. It was also continuously pressed gently with a small mortar to break up any frozen lumps which might have been present. The protein pellet was then washed four times with dry ether cooled in the acetone-carbon dioxide bath, again

with stirring. The pellet was allowed to dry out briefly and the Buchner funnel was transferred to a desiccator which was evacuated for 1 hr. at an oil pump. Careful observance of this procedure was essential to obtain successful acetone powders. Such a powder was almost white in colour. At the end of the hour, the powder was removed from the desiccator, the lumps were broken up, and the powder stored at -15°C, at which temperature it was stable for weeks. Generally, twelve glands yielded around 3.5 g. mitochondrial acetone powder.

(3) Method of Assay

An effective enzyme assay should be rapid and simple as well as sensitive and reproducible. Spectrophotometric and fluorimetric methods are generally the techniques of choice. However, the Km. of the cholesterol side-chain cleavage system is so low (2.0 μM, see fig. 26), that the products of the reaction will be present in only sub-microgram amounts. Also the first product of the reaction, pregnenolone, has no dominant chromophore in either the visible or the ultraviolet regions of the spectrum. The problem is further complicated particularly in the case of whole tissue fractions by the fact that added cholesterol substrate will be chemically indistinguishable from endogenous cholesterol and will equilibrate with it to an unknown extent. For these reasons it was decided to use a radioactive method of assay, as cholesterol - ^{14}C, labelled at either position 4 or 26, was available from the Radiochemical Centre, Amersham, at high specific activity (60 μc./mg.). Also, methods of separating and quantitating radioactive steroids were in routine use in this laboratory so it was decided to base the assay on a physical separation of the steroid products formed, followed by quantitative measurement, rather than attempt to analyse the side-chain fragments. Hence, methods of separating the various steroid products of the side-chain cleavage reaction were explored.

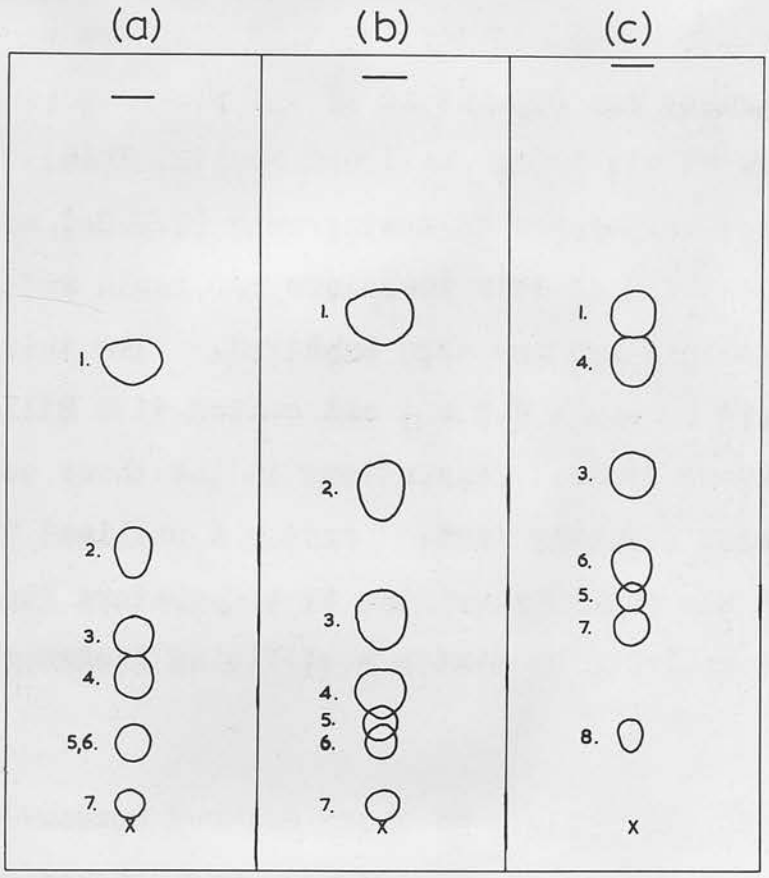


Fig.12. Thin layer solvent systems.

(a) Solvent System A. - petroleum (60-80° boiling range)/ diethyl ether/acetic acid 70:30:2. re-run in petroleum/diethyl ether/acetic acid 60:40:2.

(b) Solvent System B. - petroleum/diisopropyl ether/acetic acid 30:70:2 (2 runs)

(c) benzene/ethyl acetate/acetone 6:1:1.

1. cholesterol 2. 20α-hydroxycholesterol 3. pregnenolone 4. progesterone 5. 20α,22R-dihydroxycholesterol 6. 7-oxocholesterol 7. 11β-hydroxyprogesterone. 8. corticosterone.

Previous assay methods for this reaction used paper chromatography for separation of the products (e.g. Halkerston et al, 1961; Hall and Koritz, 1964). In this laboratory, thin-layer chromatography (T.L.C.) was in routine use, and as this technique was rapid and simple, various solvent systems were explored. The thin-layer plates were 20 cm. x 5.2 cm. and coated with Silica gel H. Fig. 12 shows typical separations in the three solvent systems most commonly used. System A utilised diethyl ether and was rather sensitive to temperature fluctuations, so it was replaced by system B utilising di-isopropyl ether.

An unusual feature of these solvent systems is the relative polarities of pregnenolone and progesterone. Generally, in T.L.C. on silica gel, steroids with the Δ^4-3-one structure are less polar than the corresponding steroids with the Δ^5-3β-ol structure. The reverse is true here in the case of pregnenolone and progesterone. This effect is discussed in Appendix 4. Steroids were detected on the T.L.C. plates by spraying the plates with either phosphomolybdic acid, phosphotungstic acid, or scintillation liquid* diluted 1:4 with methanol. These

*

The scintillation liquid consisted of 4g. PPO and 3mg. POPOP per litre toluene.

Table 2

INCUBATION MIXTURE

SUPERNATANT OF MITOCHONDRIAL SONICATE	15 mg. protein/ml.	1 ml.
or		
ACETONE POWDER EXTRACT	5 mg. protein/ml.	
PHOSPHATE BUFFER	0.1M pH 7.4	2.5 ml.
MAGNESIUM SULPHATE	200 μ moles/ml.	0.25 ml.
$NADP^+$ (in distilled water)	7.5 mg./ml.	0.5 ml.
G-6-P (in distilled water)	25 mg./ml.	0.5 ml.
G-6-P DEHYDROGENASE	1 unit/0.1 ml.	0.1 ml.
CHOLESTEROL-4-^{14}C	250,000 c./min. (6.8×10^{-3} μ moles) in 0.05 ml. acetone	0.05 ml.

system buffered at pH 7.4 with phosphate. Incubations were performed in 25 ml. Erlenmeyer flasks in air with shaking. The final volume was generally 5 ml. except in the case of incubations with whole mitochondria and microsomes, in which the final volume was 10 ml. The concentrations of components were as in Table 2, and are based on those used by Constantopoulos and Tchen (1961a). Stock solutions of cholesterol-4-^{14}C in acetone were prepared and purified routinely every two weeks to remove autoxidation products, notably 7-oxo cholesterol. These stock solutions were stored at -15°C. Incubations were commenced by addition of substrate, the remaining mixture having been previously allowed 4 min. to reach thermal equilibrium.

Extraction Procedure

Incubations were stopped by the addition of 5 ml. methanol and the mixture transferred to a stoppered centrifuge tube with two rinses, each of 2.5 ml. methanol. This transferred 98-99 per cent of the radioactivity to the centrifuge tube. The protein was sedimented and re-extracted with 5 ml. boiling ethyl acetate; 20 ml. chloroform was added to the combined extracts which were then shaken vigorously and centrifuged to separate organic and aqueous phases. The aqueous phase was removed by suction. In the case of incubations with sonicate fractions and acetone powders, only 0.5-1.0 per cent of

the radioactivity remained in the protein precipitate as determined by exhaustive refluxing with ethyl acetate. In the case of incubations with whole tissue fractions such as native mitochondria however, a second extraction with boiling ethyl acetate was necessary to achieve this degree of recovery. Finally the organic phase was taken to dryness under vacuo on a hot water bath.

Separation and Analysis

This residue was dissolved in a few drops of chloroform and applied to a thin layer plate. Repeating this operation once for each residue resulted in 90-95 per cent of the radioactivity being transferred to the plate. For most purposes this was satisfactory as enzyme activity was estimated in terms of total counts on the plate rather than total counts added per incubation.

Standard non-radioactive steroids were spotted at one side of the plate and the plates were run in solvent system A or B. The standards were visualised by spraying with a 1:4 dilution of scintillation liquid with methanol and viewing under an ultraviolet lamp, as this was the only spray which did not incur the risk of interfering with the scintillation spectrometry.

The plates were either scanned in the radioactive scanner or divided into segments for liquid scintillation counting. At first the steroids were eluted from these

Table 3

Recovery of radioactivity during extraction procedure

Radioactivity added as cholesterol-4-^{14}C	Radioactivity recovered counts/min.		Percentage of that added
232,500 c./min.	in incubation flask	20	0.01%
	in protein residue	450	0.2%
	in spotting flask	12,470	5.4%
	on T.L.C. plate	222,100	95.8%
	TOTAL		101.4%

Table 4

Comparison of Methods of estimating radioactive cholesterol bound to silica gel H

Estimations were performed in quadruplicate. The counts/mi in each sample was determined eight times by liquid scintill spectrometry. Results are presented as the mean and standa deviation of the eight determinations.

Cholesterol-4-^{14}C solution in acetone pipetted directly into scintillation liquid (10 ml.)

	1	2	3	4
counts/min.	15,547±243	14,301±230	14,050±232	13,598±318

Silica gel (corresponding to 5cm^2. of a T.L.C. plate) added to above samples which were re-counted

	1a	2a	3a	4a
counts/min.	15,505±246	14,118±256	13,943±239	13,778±290

0.1 ml. cholesterol-4-^{14}C solution spotted on T.L.C. plate; silica gel scraped directly into scintillation liquid

	2cm^2.silica gel	3cm^2.silica gel	4cm^2.silica gel	5cm^2.silica gel
counts/min.	13,694±236	13,331±243	13,992±279	14,195±344

0.1ml. cholesterol-4-^{14}C solution spotted on T.L.C. plate; eluted with $CHCl_3$/Methanol 1:1, taken to dryness and counted

	1	2	3
counts/min.	13,293±312	13,509±132	13,965±135

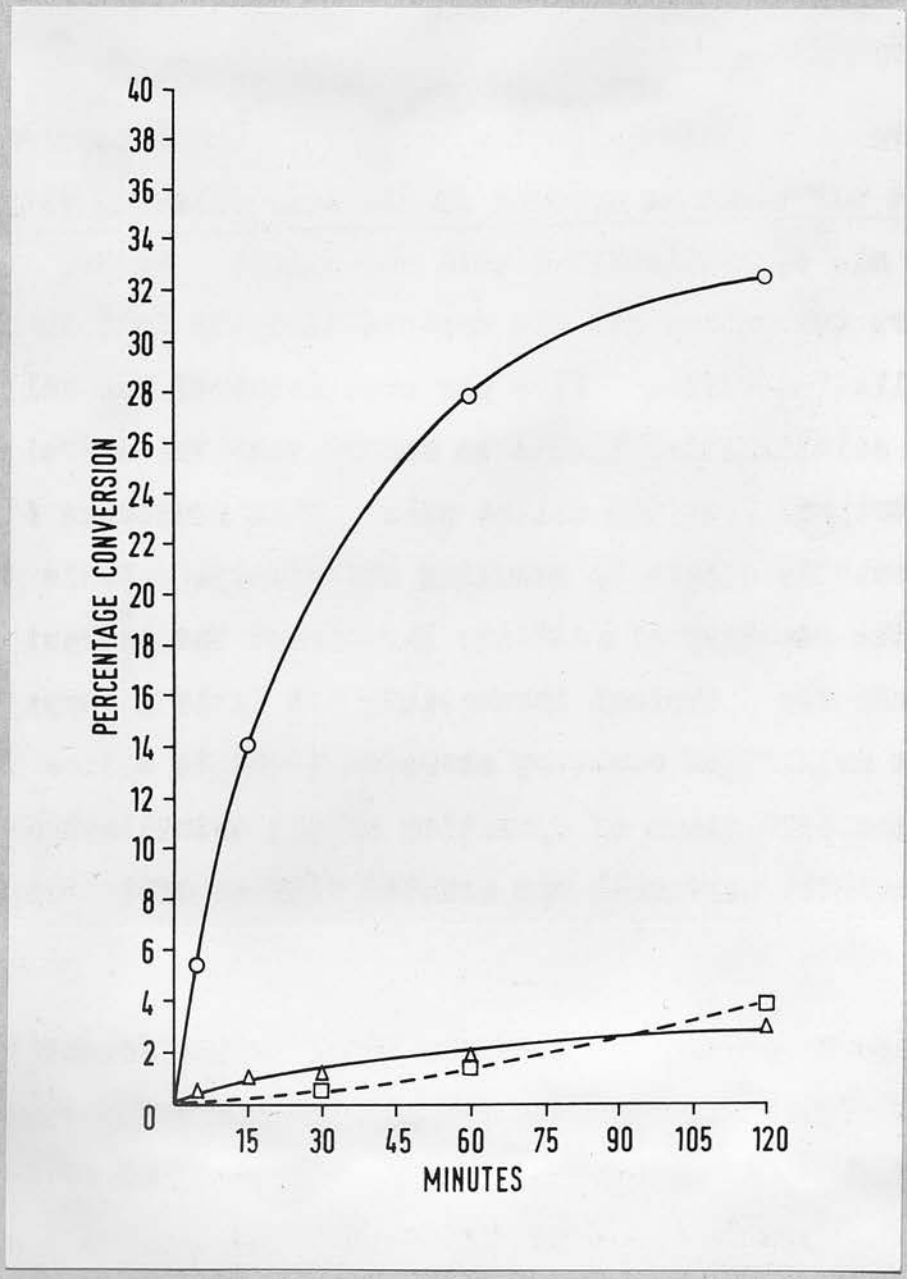


Fig.13. Time-course of metabolite formation by supernatant of mitochondrial sonicate under standard assay conditions.

o - pregnenolone

△ - progesterone

□ - polar steroids

segments with chloroform/methanol 1:1. The chloroform extract was taken to dryness in the scintillation vials, and 10 ml. scintillation liquid was added. Later, however, the silica gel was scraped directly into the scintillation vials. Five per cent methanol was added to the scintillation liquid to ensure that the steroids were stripped from the silica gel. This procedure did not result in a loss in counting efficiency. Table 3 shows the recovery of activity throughout the extraction procedure for a typical incubation, and Table 4 compares the two methods of counting steroids bound to silica gel. Under the conditions of operation of the scintillation spectrometer, carbon-14 was counted with an efficiency of 73 per cent.

Fig. 13 shows a time-course study of the formation of metabolites in which the enzyme source was the super-:natant obtained by centrifuging a mitochondrial sonicate at 105,000 xg. for 30 min. (see Chapter 3, section 5). As can be seen, the conversion rate was linear until about 15 per cent of the cholesterol was metabolised. Incubation conditions were chosen to give this conversion in about 15 min. This corresponded to 1 ml. of supernatant of mitochondrial sonicate per incubation (containing 12-15 mg. protein), the equivalent of 2 g. cortex, or 15 mg. acetone powder per incubation, the equivalent of 0.5 g. cortex. Because the conversion rate did not change with time over this period, 15 min. was the

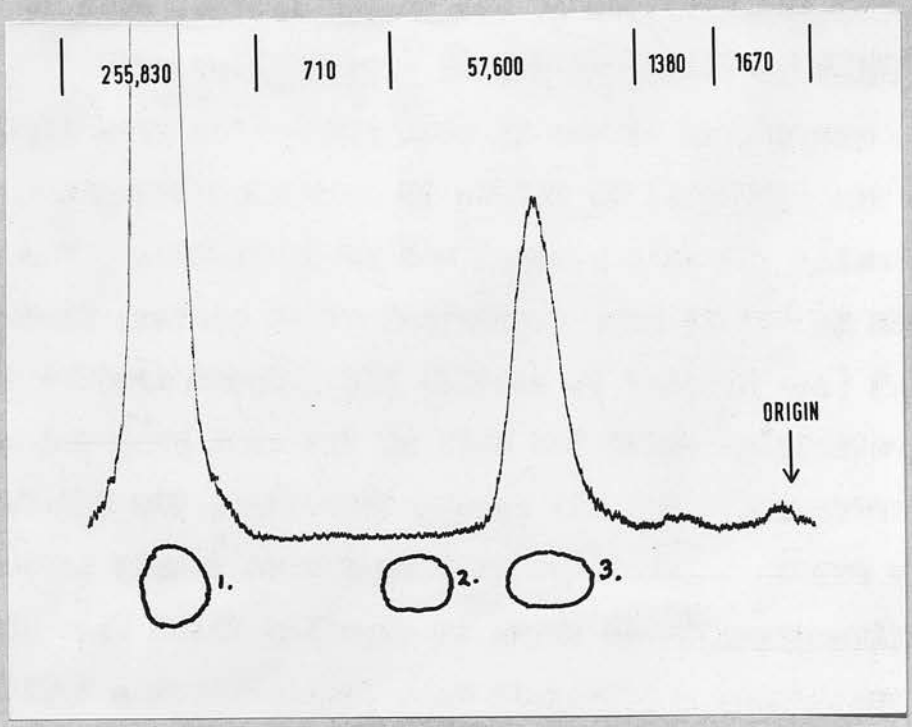


Fig.14. Thin-layer radiochromatogram of 15 min. incubation of supernatant of mitochondrial sonicate from data of fig.13, showing the segments into which the plate was divided, and the counts per minute recovered in each segment.
(solvent system A.)
1. cholesterol 2. 20α-hydroxycholesterol
3. pregnenolone.

incubation time chosen for the standard assay in studies involving the kinetics of the enzyme system, such as inhibition by carbon monoxide. Fig. 14 shows the radiochromatogram of the 15 min. incubation from fig. 13, which was separated by T.L.C. in solvent system A. Practically the only product was pregnenolone. The same applies to the 15 min. incubation of an acetone powder extract (see Chapter 3, section 4). These are the two preparations on which the bulk of the work reported here was performed. For the assay, therefore, the T.L.C. plates run in solvent system A or B were simply divided into five segments as shown in fig. 14, which also shows the counts/min. obtained in each segment from a typical plate. The rate of side-chain cleavage could therefore be expressed as counts per min. in the segment containing pregnenolone divided by the total counts per min. on the plate. With this method a complete experiment could be performed in six hours.

(4) Summary

(a) A method of homogenising bovine adrenal cortex tissue using an all-glass homogeniser and the preparation of sub-cellular fractions from this homogenate, namely mitochondria, microsomes and particle-free supernatant, are described.

(b) Techniques of ultrasonication of the adrenal cortex mitochondria, and of lyophilisation of the mitochondria with acetone, are described.

(c) A rapid and extremely sensitive method of assay of the cholesterol side-chain cleavage system is discussed. This method utilises cholesterol-4-^{14}C as substrate, and involves extraction of the steroid products and their separation by thin-layer chromatography. New thin-layer solvent systems utilising petroleum, acetic acid and an ether were developed for this purpose. Quantitative analysis of the steroid products was achieved by means of liquid scintillation spectrometry and a gas-flow radioactive scanner.

3. PRELIMINARY STUDIES ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE SYSTEM

(1) Introduction

This chapter deals firstly with the intra-cellular location of the cholesterol side-chain cleavage system. Then, general properties of the cleavage reaction are described in whole mitochondria, sonicates of mitochondria and acetone powders of mitochondria. Finally, evidence for the identity of the metabolites formed as a result of the side-chain cleavage reaction is presented.

(2) Sub-cellular localisation of the cholesterol side-chain cleavage system

Experiments were set up to establish in which cell fraction the cholesterol side-chain cleavage activity of bovine adrenal cortex resided. Incubations were set up containing mitochondria or microsomes equivalent to 3 g. cortex in a final volume of 10 ml. In the incubations stated, 3 ml. 105,000 xg. supernatant corresponding to 1 g. cortex, was added instead of 3 ml. buffer. The concentration of components was as in Table 2. The total amount of cholesterol substrate, however, was the same as in Table 2. Incubations were performed at 37°C and the incubation time was 2 hr. The results for duplicate experiments were:-

Incubation		Conversion to pregnenolone and progesterone	Conversion to polar steroids	Total conversion
Washed mitochondria	(1)	4.0%	3.4%	7.4%
	(2)	1.3%	3.5%	4.8%
Washed microsomes	(1)	0.2%	< 0.1%	0.3%
	(2)	0.2%	< 0.1%	0.3%
Washed microsomes plus 105,000 xg. supernatant	(1)	0.5%	0.3%	0.8%
	(2)	0.5%	0.2%	0.7%

It can be seen that the most active fraction was the mitochondria. This is in keeping with most published data (e.g. Halkerston et al, 1961; Hall and Koritz, 1964). Microsomes alone contained no appreciable activity, but microsomes plus supernatant did possess some activity. This could be due to partial liberation of enzymes from the mitochondria as a result of the homogenisation procedure. It was decided to examine the cholesterol side-chain activity of the mitochondria in greater detail.

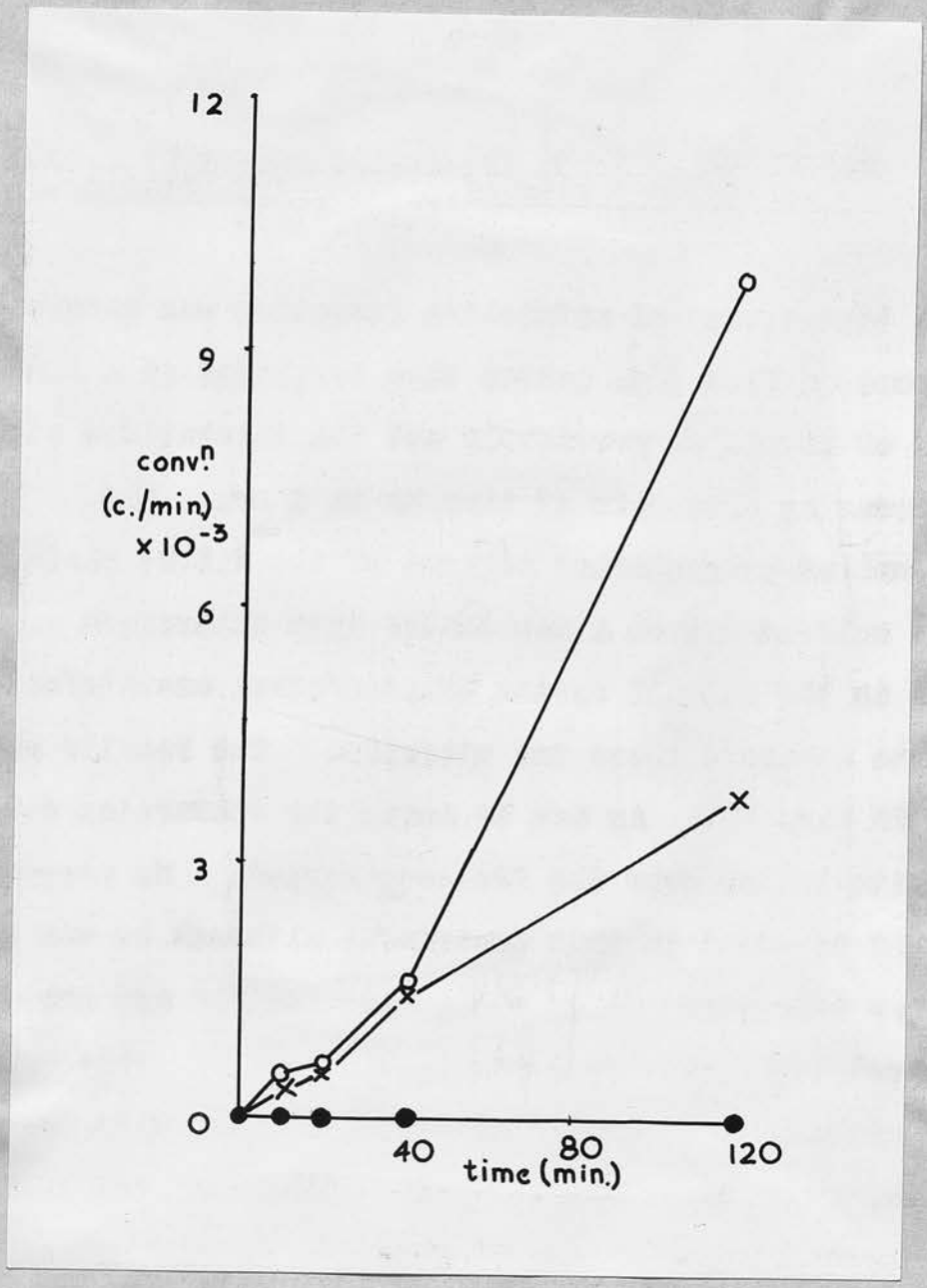


Fig.15. Time-course of metabolite formation by whole mitochondria.

o - pregnenolone

• - progesterone

x - polar steroids

(3) Characteristics of the cholesterol side-chain cleavage system of native mitochondria

A time-course of metabolite formation was carried out. Mitochondria from 3 g. cortex were incubated in a final volume of 10 ml. as previously and the incubations stopped at increasing intervals of time up to 2 hr. The pregnenolone-progesterone segment of the T.L.C. plates run in solvent system A was eluted with chloroform and re-run in the solvent system benzene/ethyl acetate/acetone 6:1:1 to separate these two steroids. The results are shown in fig. 15. As can be seen, the conversion rate was quite linear over the two-hour period. No progesterone could be detected in this experiment although it was found in other experiments with whole mitochondria and sub-mitochondrial fractions (e.g. fig. 13). Pregnenolone and polar steroids were formed at about the same rate for 40 min., after which pregnenolone accumulated relative to the further metabolites. As progesterone did not accumulate, this probably means that the 3β-ol dehydrogenase was becoming less active with time.

An experiment was then conducted to compare the conversion rates when the system was fortified with NADPH-generator and when fortified with succinate, following reports that succinate was a better source of electrons for the cholesterol side-chain cleavage reaction than NADPH (Koritz, 1966). Sodium succinate was added to give the same final concentration in the incubation

as normally used for $NADP^+$, namely 1mM. The results were:-

Incubation	Conversion to pregnenolone and progesterone	Conversion to polar steroids	Total conversion
NADPH-generator	4.2%	3.1%	7.3%
Sodium succinate	10.1%	0.7%	10.8%
No addition	1.1%	not significant	1.1%

Thus, succinate appeared to be a better source of reducing equivalents for the cleavage reaction than was NADPH. Also with succinate, no polar steroids were formed. With no additions, the side-chain cleavage activity was greatly reduced.

As the primary aim of this work was to study the cholesterol side-chain cleavage system from a mechanistic point of view rather than in relation to the cell as a whole, methods of solubilising the enzyme system were explored.

As several reports exist in the literature that cholesterol side-chain cleavage activity can be extracted with buffer from mitochondria which have been lyophilised with acetone (e.g. Constantopoulos and Tchen, 1961a), the characteristics of acetone powders of adrenal cortex mitochondria were explored.

(4) Acetone Powders of Adrenal Cortex Mitochondria

Test of Activity

Acetone powders prepared as described in Chapter 2, section 2, were suspended in phosphate buffer by gentle hand homogenisation. As an initial test of side-chain cleavage activity, incubations were set up using acetone powders from 6 g. tissue in a total volume of 10 ml. The incubations were stopped at different times. The T.L.C. plates run in solvent system A were divided as in fig. 14. The results were as follows:-

Incubation time (min.)	-	0.0	0.5	10	20	40
Conversion to pregneno-:lone and progesterone	-	0.5%	18%	18.4%	8.4%	1.6%
Conversion to polar steroids	-	0.5%	2.0%	31%	57%	81%
Total conversion	-	1.0%	20%	49%	65%	83%

Thus, it can be seen that the acetone powder was many times more active than native mitochondria. This could be due to removal of endogenous cholesterol which may compete with the added labelled cholesterol for the enzyme site, or disruption of the mitochondrial structure resulting in greater accessibility of the enzyme system to the added substrate and NADPH, or both of these. Certainly the acetone powder contained very little

endogenous cholesterol, as seen when viewing the plates sprayed with the dilute scintillator, whereas native mitochondria contained considerable quantities. The experiment also demonstrated the initial conversion to pregnenolone and progesterone, with subsequent conversion of these to more polar products. Incubation conditions were subsequently modified to those stated in Chapter 2, section 3, with 15 mg. acetone powder incubated in a total volume of 5 ml. The counts along the plate for a typical 15 min. incubation were:-

Segment	-	1	2	3	4	5
Counts/min.	-	25,220	1,670	15,520	350	212,000

with the segments as in fig. 14. The conversion was 16.8 per cent and over half the counts were in the polar steroid segment.

Extraction

There are many reports in the literature indicating the ability of phosphate buffer to extract cholesterol side-chain cleavage activity from the mitochondrial acetone powder, for example, Constantopoulos and Tchen (1961a). Therefore, the ability of phosphate buffers of different molarities to extract the activity from the acetone powder was investigated. 15 mg. powder was homogenised with 7.5 ml. of the appropriate buffer and centrifuged at 105,000 xg. for 60 min. to give a pale

brown, clear supernatant. This was incubated in a final volume of 10 ml. to give the following results:-

Incubation	Buffer	Counts/min. T.L.C. plate segments				
		1	2	3	4	5
1 (Control)	0.1M(P)	20,900	6,630	36,000	1,600	324,200
2	0.5M(P)	1,090	210	2,150	630	274,600
3	0.1M(P)	1,580	600	53,980	2,160	312,400
4	0.02M(P)	1,800	510	80,850	1,110	294,600

Incubation 1 was the control without extraction. The results show that with 0.1M phosphate and 0.02M phosphate buffer, the side-chain cleavage activity was extracted quantitatively from the acetone powder, and 0.02M phosphate apparently caused stimulation of activity. Also, the activity was confined to the pregnenolone-progesterone segment. Scanning a plate run in solvent system B revealed that there was only a trace of labelled progesterone in this segment, indicating that the 3β-ol dehydrogenase and/or the Δ^5-3-ketosteroid isomerase remained in the pellet after the centrifugation.

The dependency of the ability of phosphate buffer to extract the activity upon the amount of powder added per ml. buffer was then investigated. It was found that

with 0.02M phosphate, increasing the amount of powder per ml. buffer decreased the ability of the buffer to extract the activity from the powder:-

mg. powder/ml. buffer	Percentage conversion in 15 min.
2.5	6.5
7.5	3.4

However, with 0.1M phosphate the activity could be extracted quantitatively from the powder up to a concentration of 15 mg./ml. buffer, which was the highest tested. Therefore, acetone powders were routinely extracted with 0.1M phosphate buffer at a concentration of 15 mg. powder/ml. buffer, and 1 ml. extract was incubated in a final volume of 5 ml. Such an extract had a protein content of 4.5 mg./ml. as measured by the Biuret Method of Layne (1957).

Stability of acetone powders

As mentioned previously, acetone powders could be stored at -15°C for several weeks without activity loss. They could also be kept at room temperature for 4 hr. without loss of activity. Repeated cooling to -15°C and warming back to room temperature also did not affect the activity.

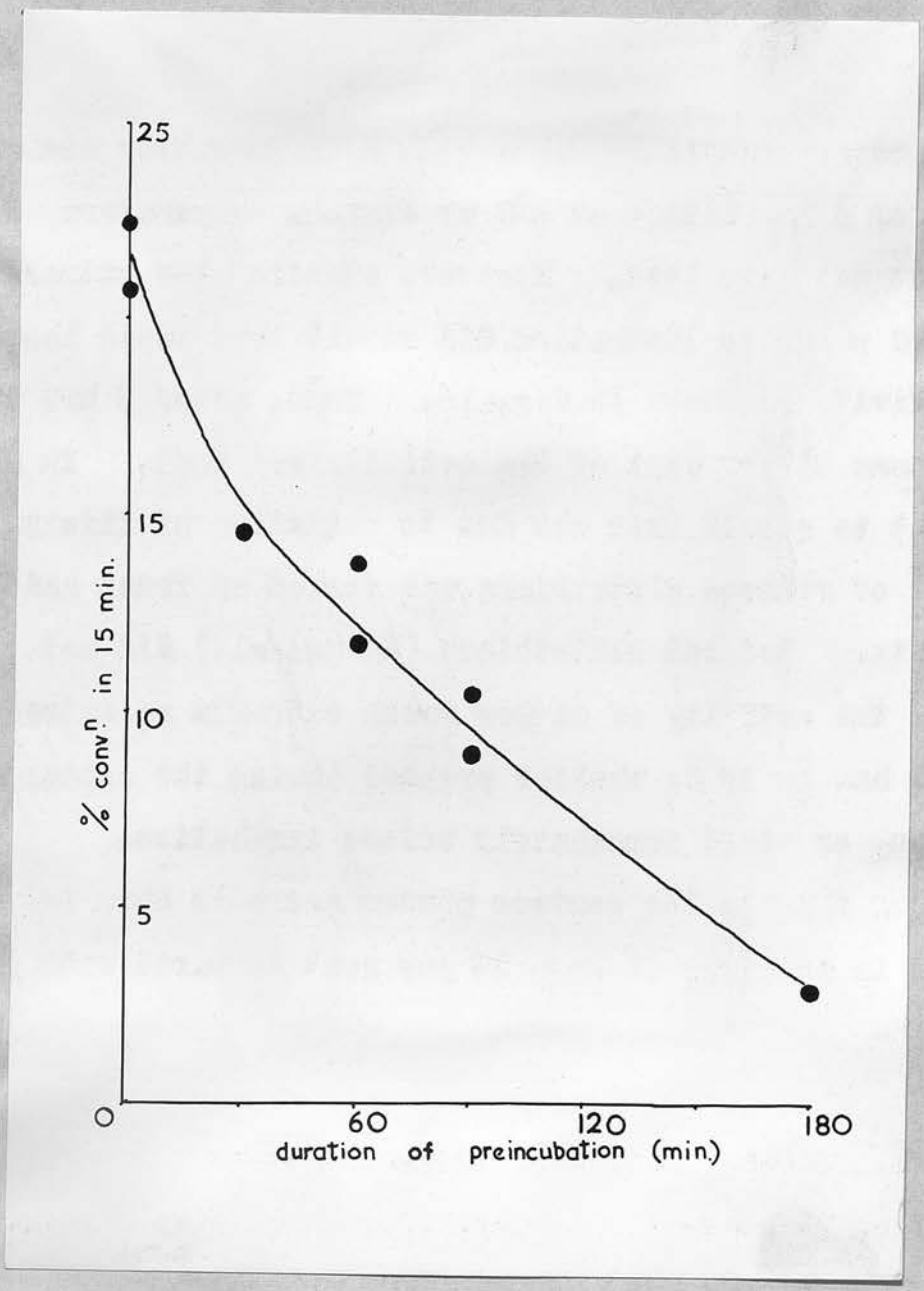


Fig.16. Effect of preincubation at 37°C on the cholesterol side-chain cleavage activity of acetone powder extract. After preincubation, samples were withdrawn and incubated at 37° for 15 min. with cholesterol-4-^{14}C under standard assay conditions.

Acetone powder extracts with 0.1M phosphate could be left for 5 hr. either at 4°C or at room temperature without activity loss. However, standing the extract at 37°C prior to incubation did result in a rapid loss of activity as shown in fig. 16. Thus, after 3 hr. at 37°C some 85 per cent of the activity was lost. In an attempt to see if this was due to oxidation of thiols, the effect of reduced glutathione was tested on fresh and aged extracts. Reduced glutathione (60 µg./ml.) did not affect the activity of either fresh extracts or extracts aged 2 hr. at 37°C, whether present during the ageing process, or added immediately before incubation. Freezing and thawing acetone powder extracts once led to a loss in activity of some 20 per cent compared with the control.

Optical spectrum of acetone powder extract

Fig. 17 shows the reduced carbon monoxide difference spectrum of the acetone powder extract reduced with dithionite. The chromophore at 450 mµ is characteristic of cytochrome P-450. The chromophore at 420 mµ could have been due to contaminating haemoglobin and the degradation form of P-450, known as P-420.

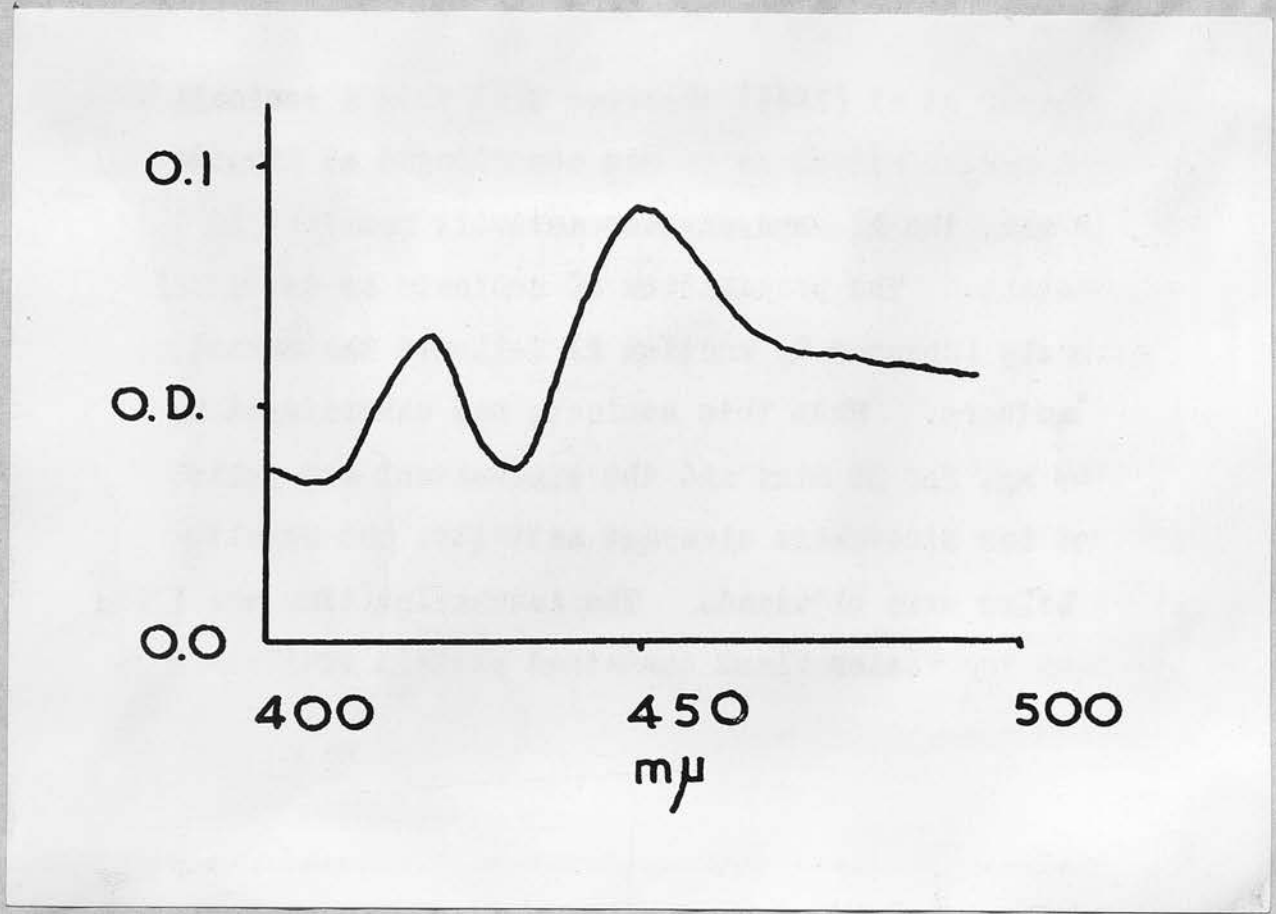


Fig.17. Dithionite - reduced carbon monoxide difference spectrum of acetone powder extract, containing 5 mg. protein per ml.

(5) Sonicates of Adrenal Cortex Mitochondria

Cooper et al (1965) observed that when a sonicate of adrenal cortex mitochondria was centrifuged at 105,000 xg. for 30 min. the 11β-hydroxylase activity remained in the supernatant. The preparation of sonicate as described previously (Chapter 2, section 2) followed the method of these authors. When this sonicate was centrifuged at 105,000 xg. for 30 min. and the supernatant and pellet assayed for side-chain cleavage activity, the results shown below were obtained. The incubation time was 1 hr. and each incubation flask contained protein equivalent to 1 g. cortex.

Incubation	Percentage Conversion	
	pregnenolone + progesterone	polar steroids
Supernatant	29	3
Pellet	1	0
Supernatant + pellet	18	26

This showed that the side-chain cleavage activity also resided in the supernatant of the mitochondrial sonicate and that most of the radioactivity was in the segment of the plate containing pregnenolone and progesterone. A radiochromatogram of a T.L.C. plate from such an

incubation run in solvent system B showed that almost all the radioactivity was in the position corresponding to the pregnenolone standard. When the pellet was added to the incubation however, more than half the radioactivity appeared in the polar steroid fraction. A similar situation was found in acetone powders, indicating that in both cases the 3β-ol dehydrogenase and/or the Δ^5-3-ketosteroid isomerase activity resided chiefly in the pellet obtained after centrifugation. Some of the other hydroxylases which must be present to form the polar steroids may also have been in this pellet, although the 11β-hydroxylase was presumably in the supernatant in both cases, (Suzuki and Kimura, 1965; Cooper et al, 1965). It seems somewhat unlikely that these enzymes were present entirely as contaminants due to imperfect cell fractionation, as the mitochondria were washed twice with isotonic potassium chloride (see fig. 56). Also the total conversion in the supernatant and pellet together was greater than that in the supernatant alone. This could be due to the inhibitory nature of the accumulated pregnenolone in the incubation with the supernatant of the sonicate alone (Koritz and Hall, 1964).

Effect of Freezing

The supernatant of mitochondrial sonicate could be stored for several weeks at -15°C without loss of activity.

Time Course of Incubations with supernatant of Mitochondrial Sonicate

As discussed previously, incubation conditions were chosen to give a conversion of 15 per cent in 15 min. This corresponded to 1 ml. supernatant of mitochondrial sonicate per incubation (obtained from about 2 g. cortex). This amount was used throughout and was incubated in a final volume of 5 ml. The protein content was 12-15 mg./ml. depending on the batch, as measured by the Biuret Method of Layne (1957). A time-course study of such incubations is shown in fig. 13. It can be seen that with time, small amounts of progesterone and polar steroids did accumulate.

Supernatant of mitochondrial sonicate, although not as active in terms of wet weight tissue as acetone powder extract, was considerably more active than native mitochondria. This again could be due to loss of endogenous cholesterol and/or increased accessibility of the enzymes to substrate and NADPH.

Effect of $NADP^+$ concentration on activity of supernatant of Mitochondrial Sonicate

When the NADPH-generating system was omitted from the incubation, the cholesterol side-chain cleavage activity was completely abolished. When, however, only the $NADP^+$ was omitted, the activity was reduced but not

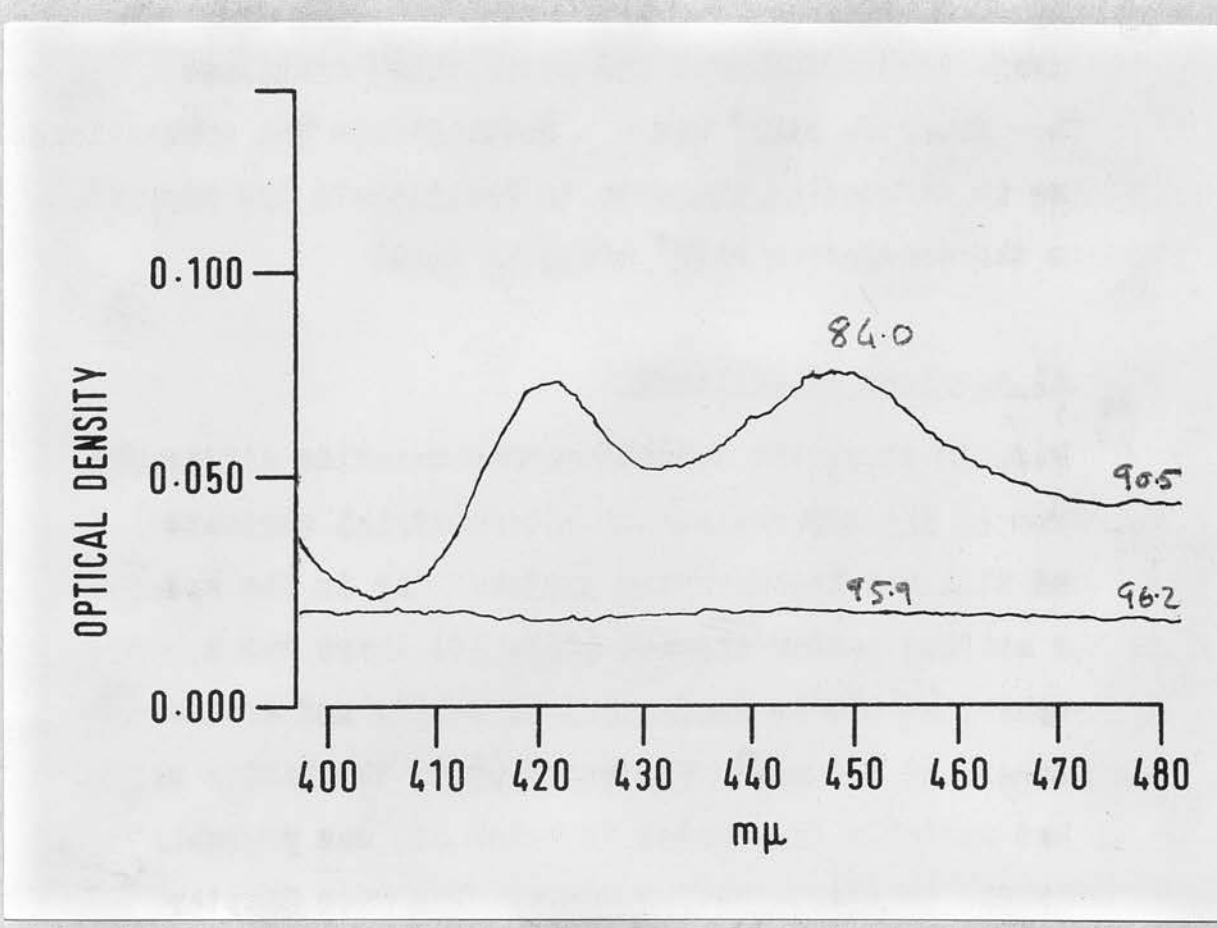


Fig.18. NADPH - reduced carbon monoxide difference spectrum of supernatant of mitochondrial sonicate containing 15 mg. protein per ml.

abolished, indicating that the preparation contained $NADP^+$. However, $NADP^+$ was not omitted from the incubations, because in subsequent attempts to fractionate the enzyme system the endogenous $NADP^+$ might be lost.

Optical spectrum of sonicate

Fig. 18 shows the reduced carbon monoxide difference spectrum of the supernatant of mitochondrial sonicate reduced with NADPH-generating system. As in the spectrum of the acetone powder extract (fig. 17) there was a chromophore at 450 mμ indicative of P-450, and another chromophore at 420 mμ. The quantity of the latter was somewhat variable from batch to batch and was probably largely due to contaminating haemoglobin (see Chapter 7, section 7).

As the published work on the 11β-hydroxylase was performed with supernatant of mitochondrial sonicate, namely carbon monoxide inhibition and its light reversal, and isolation and purification of the system, this was the source of enzyme used for many of the comparable investigations on the cholesterol side-chain cleavage system reported here.

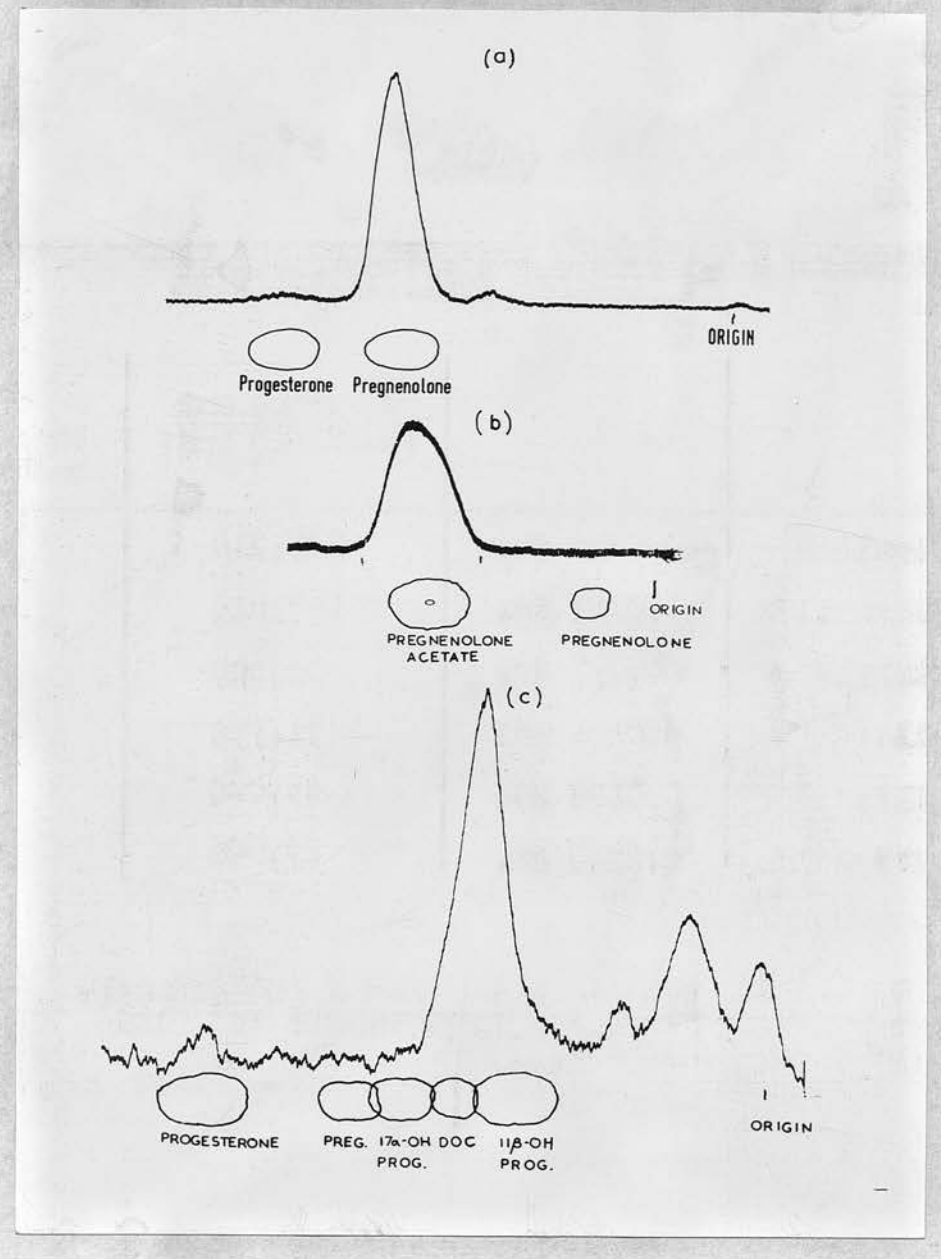


Fig.19. Thin - layer radiochromatograms of metabolites.

(a) pregnenolone and progesterone.

(b) pregnenolone acetate.

(c) polar steroids.

(a) and (c) - solvent systems benzene/ethyl acetate/ acetone 6:1:1.

(b) solvent system benzene/ethyl acetate 10:1.

Table 5

Recrystallization of pregnenolone and progesterone to constant specific activity

	mass	pregnenolone counts/10 min.	specific activity counts/10 min./µg.
Precrystallization	0.0348 gm.	205,710	5.92
1st recrystallization	0.0281 gm.	127,010	4.52
2nd recrystallization	0.0227 gm.	92,990	4.09
3rd recrystallization	0.0181 gm.	71,950	3.98
4th recrystallization	0.0176 gm.	65,020	3.70
5th recrystallization	0.0219 gm.	87,200	3.98

Pregnenolone was recrystallized from methanol. Samples were taken for weighing and the same sample counted.

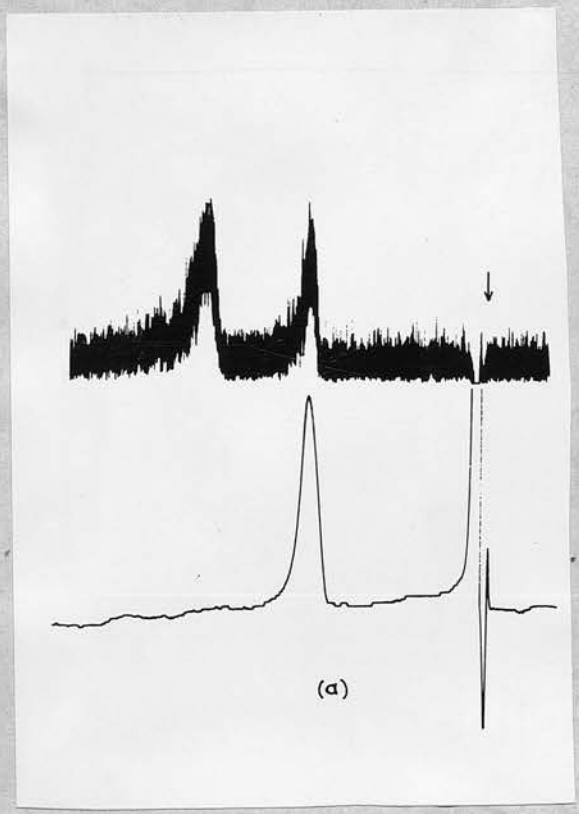
	E_{242}	progesterone counts/30 min.	Specific activity counts/30 min./µg.
recrystallization	0.514	5435	193
st recrystallization	0.361	3787	186
nd recrystallization	0.245	2575	192

Progesterone was recrystallized from methanol. Mass was calculated from E_{242} using a calibration graph.

(6) Identification of Metabolites

A radiochromatogram of a T.L.C. plate from a typical incubation run in solvent system B showed radioactive components which behaved as pregnenolone, progesterone and more polar products. The peak corresponding in polarity to pregnenolone also behaved like pregnenolone in the solvent system benzene/ethyl acetate/acetone 6:1:1 as in fig. 19a, (see fig. 12 for typical separations). Acetylation with acetic anhydride/pyridine 1:1 on a hot water bath for 1 hr. gave a product which behaved like pregnenolone acetate in the solvent system benzene/ethyl acetate 10:1 (fig. 19b). The material also behaved like pregnenolone when subjected to gas liquid chromatography (G.L.C.) using a Pye Argon Radiochromatogram on columns containing 1 per cent XE-60 (fig. 20b) and a mixture of 1 per cent JXR and 2 per cent XE-60 (fig. 20a), both on 100-120 mesh gas chrom. Q (Applied Science Laboratories). Finally, the material was crystallised to constant specific activity with carrier pregnenolone (Table 5).

The peak corresponding to progesterone in solvent system B also behaved like progesterone in the solvent system benzene/ethyl acetate/acetone 6:1:1 (fig. 19a) and when subjected to G.L.C. using 1 per cent XE-60 on gas chrom. Q (fig. 20c). It was recrystallised to constant specific activity with carrier progesterone (Table 5).



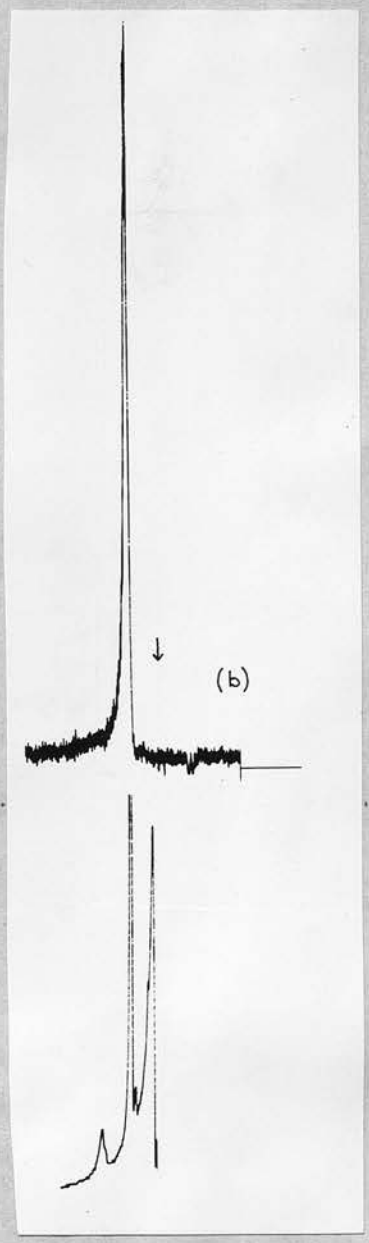


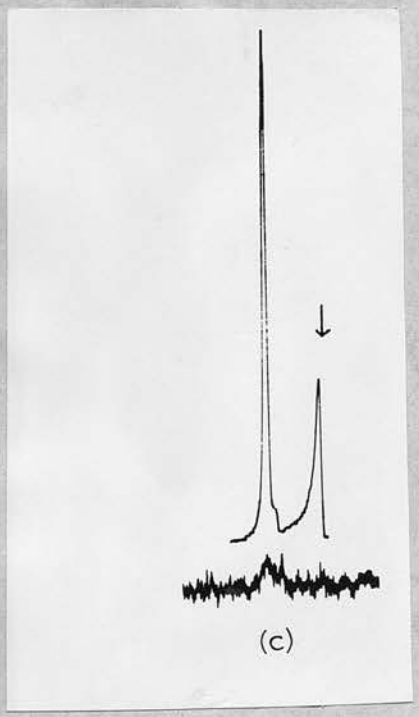
Fig.20. Gas - liquid radiochromatograms of metabolites.

(a) Standard non-radioactive pregnenolone and radioactive product from incubation, identified as pregnenolone, along with standard cholesterol-4-^{14}C.

column 1%JXR and 2%XE60 on gas-chrom Q. Temp.226°C. Argon press. 10Kg.cm.$^{-2}$ CO_2 flow rate 2 ml.min.$^{-1}$

(b) Standard non-radioactive pregnenolone and radioactive product from incubation identified as pregnenolone.

column 1%XE60 on gas-chrom Q. Temp.212°C.



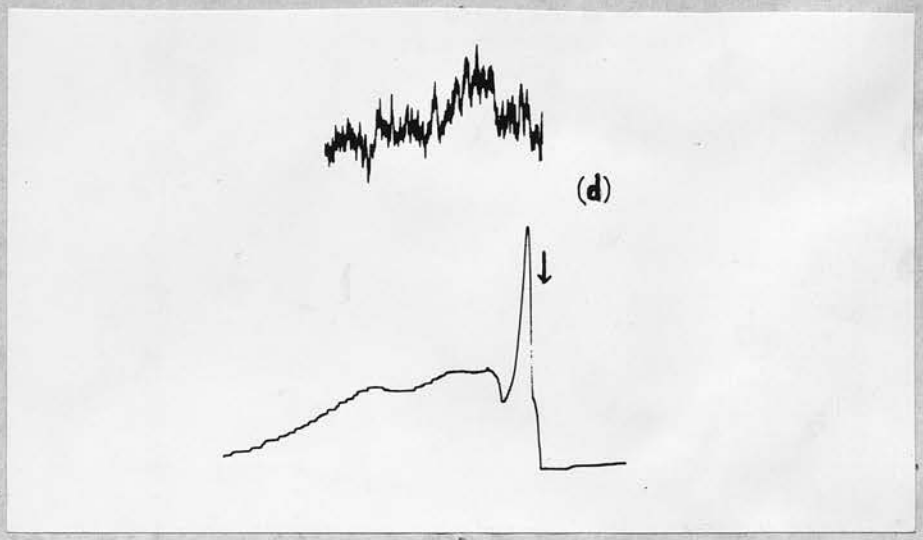


Fig.20. contd.

(c) Standard non-radioactive progesterone and radioactive product from incubation identified as progesterone. Column conditions as in (b).

(d) Standard non-radioactive 21-hydroxyprogesterone and radioactive product from incubation identified as 21-hydroxyprogesterone. Column conditions as in (c).

When the more polar material was run in the solvent system benzene/ethyl acetate/acetone 6:1:1, the major product behaved not unlike ~~like~~ 21-hydroxyprogesterone (fig. 19c). There were three more polar products. The product behaving not unlike ~~like~~ 21-hydroxyprogesterone when chromatographed on the 1 per cent XE-60 column did not give a sharp peak but rather a long, broad smear. However, standard 21-hydroxyprogesterone behaved in an identical manner (fig. 20d). The remaining more polar products were not identified.

The enzymes responsible for converting pregnenolone to progesterone and 21-hydroxyprogesterone, namely the 3β-ol dehydrogenase and Δ^5-3-ketoseroid isomerase and the 21-hydroxylase are believed to be microsomal enzymes, yet progesterone is frequently formed in mitochondrial preparations (Morrison et al, 1965). Halkerston et al (1961) also found that cholesterol was metabolised to progesterone and more polar steroids by mitochondrial preparations from adrenal cortex. The 18- and 11β-hydroxylases are, however, known to be mitochondrial, yet surprisingly no 11β-hydroxyprogesterone appeared to be formed.

(7) Summary

(a) Using the assay method described in 'Experimental 'Procedures', the cholesterol side-chain cleavage activity of bovine adrenal cortex was found to reside chiefly in the mitochondrial fraction.

(b) This cleavage activity was present in mito-:chondria which had been subjected to ultracentrifugation and acetone-drying at a level many times greater than that present in native mitochondria. The activity could be extracted from the sonicated mitochondria by centri-:fuging at 105,000 xg. for 30 min. and from an acetone powder of mitochondria by homogenising the powder in phosphate buffer and centrifuging at 105,000 xg. for 60 min. Some general properties of these preparations are described.

(c) The products of the side-chain cleavage reaction were pregnenolone, progesterone and more polar products. One of these more polar products was tentatively identified as 21-hydroxyprogesterone. 11β-hydroxyprogesterone did not appear to be formed.

(d) In supernatant of mitochondrial sonicate and acetone powder extracts, very little progesterone and polar steroids were formed, but in whole mitochondria, sonicates and acetone powders, these were formed in much greater quantities.

4. STUDIES ON INTERMEDIATES BETWEEN CHOLESTEROL AND PREGNENOLONE

(1) Introduction

As has been discussed in the General Introduction, evidence for the involvement of 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol as intermediates in the side-chain cleavage of cholesterol is conflicting. Claims for the isolation of these compounds from incubations are no more frequent than claims of failure to isolate them.

Our assay technique for the separation of the metabolites of cholesterol is particularly well suited to studying this problem as:-

(1) The thin-layer solvent systems A and B separate 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol from cholesterol, pregnenolone, progesterone and other products of the side-chain cleavage reaction.

(2) The thin-layer radiochromatogram scanner is useful for detection of small radioactive peaks which may be partially obscured by an adjacent large peak.

(3) Very small radioactive peaks can be estimated accurately by liquid scintillation counting of segments of the thin-layer chromatograms by extending the counting time.

As can be seen from a radiochromatogram of a typical incubation (fig. 14), there was no sign of any radioactive material behaving like 20α-hydroxycholesterol. The position with respect to 20α,22R-dihydroxycholesterol is

more difficult because it overlaps with 7-oxo-cholesterol which is always present in traces, but nevertheless there was no formation of 20α,22ξ-dihyroxycholesterol of the same order of magnitude as that of pregnenolone. This is in agreement with other reports in which these compounds have not been detected in the absence of a trap or inhibitor. Hall and Koritz (1964) have suggested these compounds do not occur free in the normal system but are enzyme-bound, the first free product being pregnenolone.

(2) A Time-Course Study of the early events in the Side-Chain Cleavage Reaction

If 20α-hydroxycholesterol and 20α,22ξ-dihydroxy-:cholesterol are enzyme-bound intermediates, they should nevertheless occur in amounts corresponding to the molar quantity of the respective enzyme-steroid complex present, and should be formed before pregnenolone in a time-course study. Hence there should be a lag-time phase before pregnenolone can be detected.

The possibility remained that 20α-hydroxycholesterol was present in the incubations, but the amounts present were below the limit of sensitivity of the radioactive scanner. A far more sensitive means of detection would be to divide the thin-layer plates into 0.5 cm. strips, each of which was counted by liquid scintillation spectrometry. A time-course study was carried out using this method. Incubations were performed from zero-time to 10 min., both at 37°C and 20°C. For the zero-time incubation, methanol was added to the incubation before the substrate. Each of the 0.5 cm. segments was counted for 5 min. In this way, any segments containing more than 100 counts per min. above background would be significant, that is, a concentration of approximately 5×10^{-4} µM. in the incubation, considering only products formed from the added labelled cholesterol, and not any which might be formed from endogenous sources.

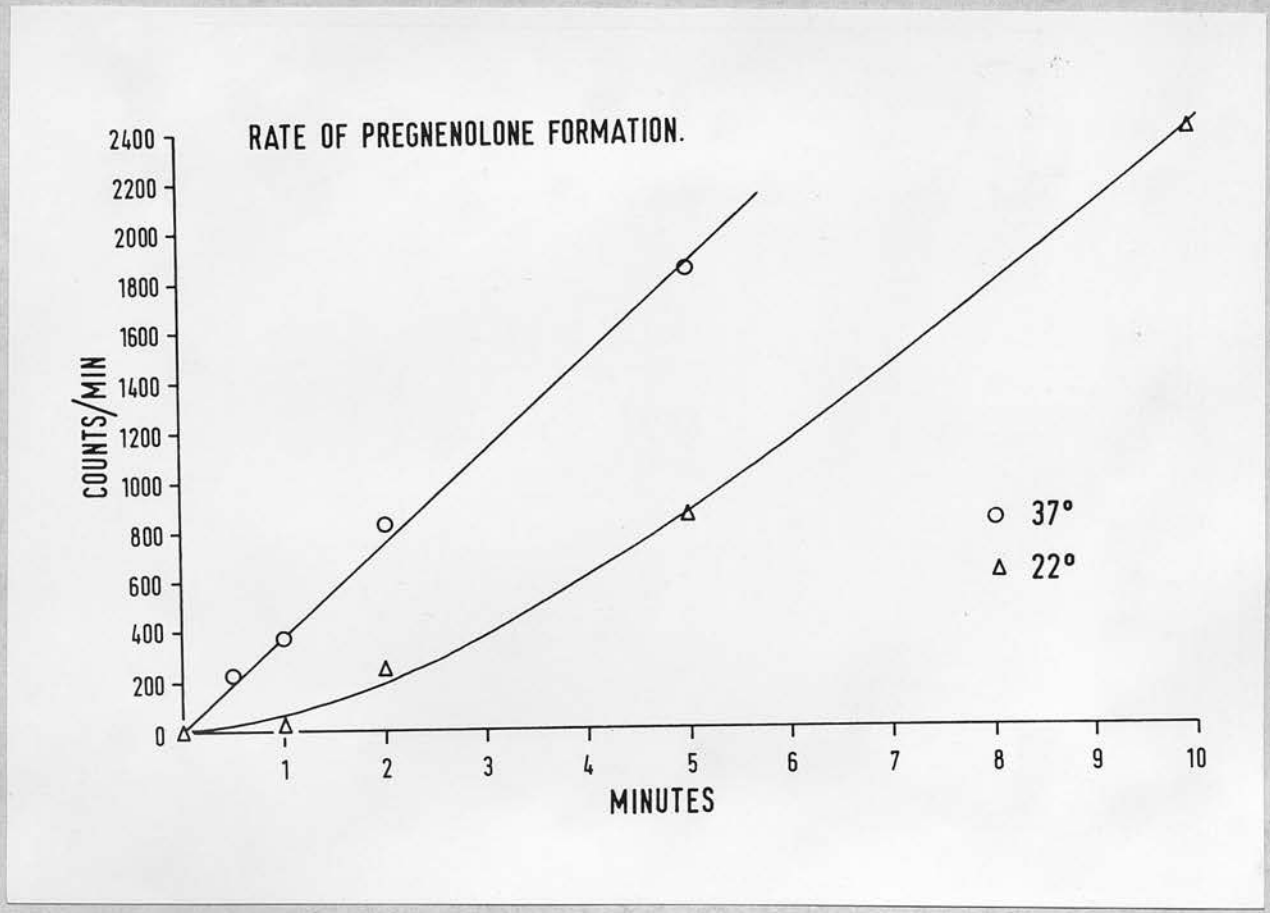


Fig.21. Time-course of pregnenolone formation.
(a)

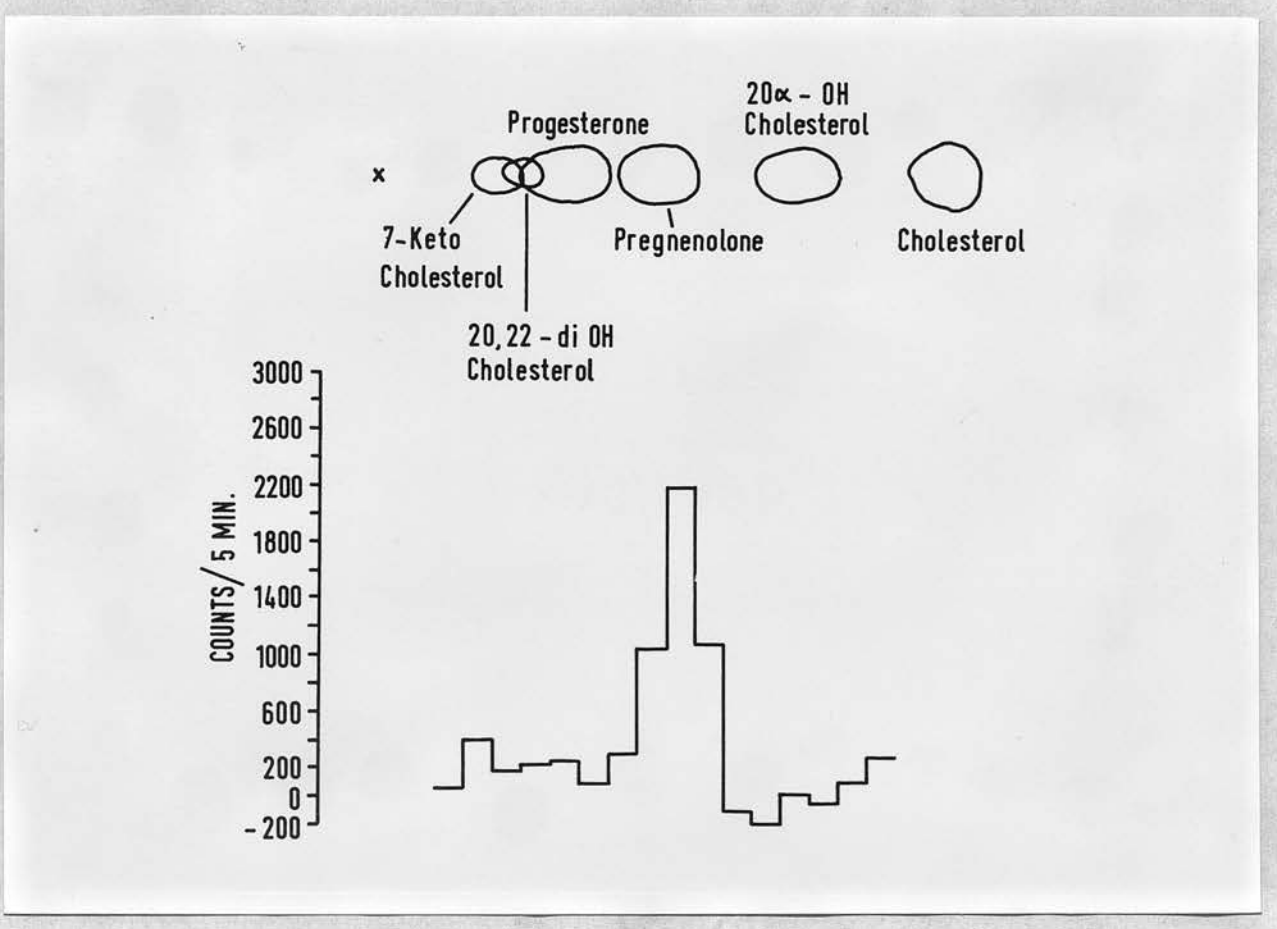


Fig.21. Thin-layer radiohistogram of 20°C - 5 min. (b) incubation from (a). Solvent system B. Enzyme source was supernatant of mitochondrial sonicate.

The "background" radioactivity in an average scintillation vial was only 50 counts/min., but the "background" radioactivity in the segments of a T.L.C. plate was somewhat variable due to small amounts of autoxidation products and other minor impurities which became significant at this level of detection, even in the zero-time control.

Fig. 21a shows the results for both temperatures. The relative rates are not comparable as a greater quantity of enzyme preparation was used in the case of the 20°C incubations. At 37°C there was no appreciable lag in the formation of pregnenolone. At 20°C however, there was a lag of some 30 sec. before pregnenolone could be detected. Fig. 21b shows the radioactivity histogram for the 20°C - 5 min. incubation corrected for small impurities and autoxidation products in the zero-time control. As can be seen, pregnenolone was the only product. No other product was found on any of the plates. Therefore, if any 20α-hydroxycholesterol or 20α,22ξ-dihydroxycholesterol were present, their concentrations were below 5×10^{-4} μM. This means that the concentration of enzyme-steroid complex for these compounds is very low. As the apparent Km. is 2.0 μM. (fig. 26), this would require that the concentration of enzyme-steroid complex be some 4,000 times lower than this.

In order to check that 20α-hydroxycholesterol, 20α,22ξ-dihydroxycholesterol or some other intermediate

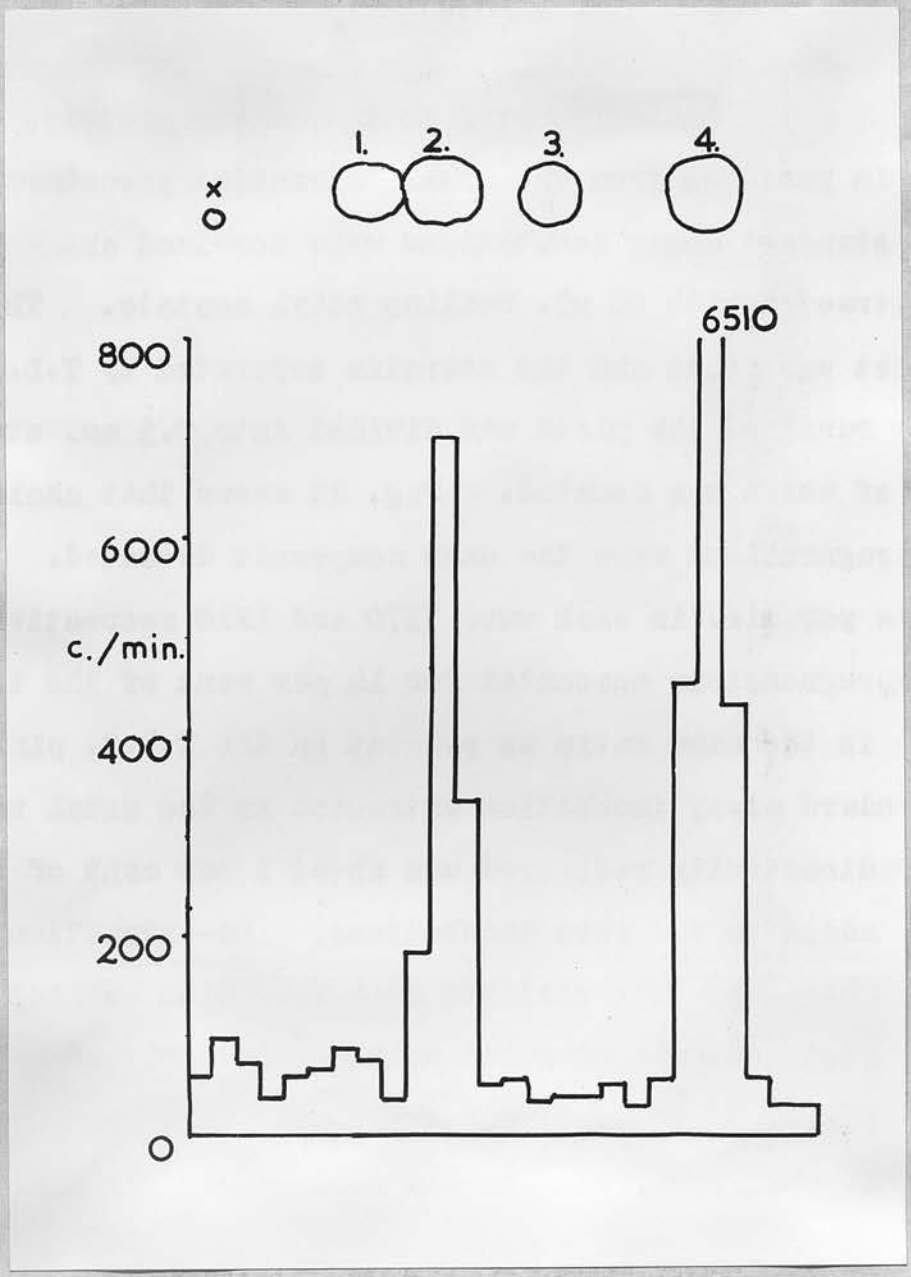


Fig.22. Thin-layer radiohistogram of protein residues from five incubations extracted with ethyl acetate. Solvent system B.

1. progesterone 2. pregnenolone 3. 20α-hydroxy-cholesterol. 4. cholesterol.

was not preferentially bound to the enzyme protein, the protein residues from the usual extraction procedure of five standard assay incubations were combined and re-extracted with 20 ml. boiling ethyl acetate. The extract was dried and the steroids separated by T.L.C. After running, the plate was divided into 0.5 cm. strips each of which was counted. Fig. 22 shows that cholesterol and pregnenolone were the only compounds detected. The counts per min. in each were 7370 and 1210 respectively, i.e. pregnenolone accounted for 14 per cent of the total, which is the same ratio as present on the T.L.C. plate of a standard assay incubation extracted in the usual way. The radioactivity recovered was about 1 per cent of the total added to the five incubations. Re-extraction of the combined protein residues once more with boiling ethyl acetate again revealed only cholesterol and pregnenolone in the same ratio. This time the radio-:activity recovered was exactly 1000 counts per min.

The combined protein residues were then combusted and the carbon dioxide formed trapped in 5 ml. Hyamine. 1 ml. of this solution was assayed for carbon-14 by liquid scintillation spectrometry and was found to contain 115 counts/min. Thus there was no material present in significant amounts which was firmly bound to the enzyme and not extractable with ethyl acetate.

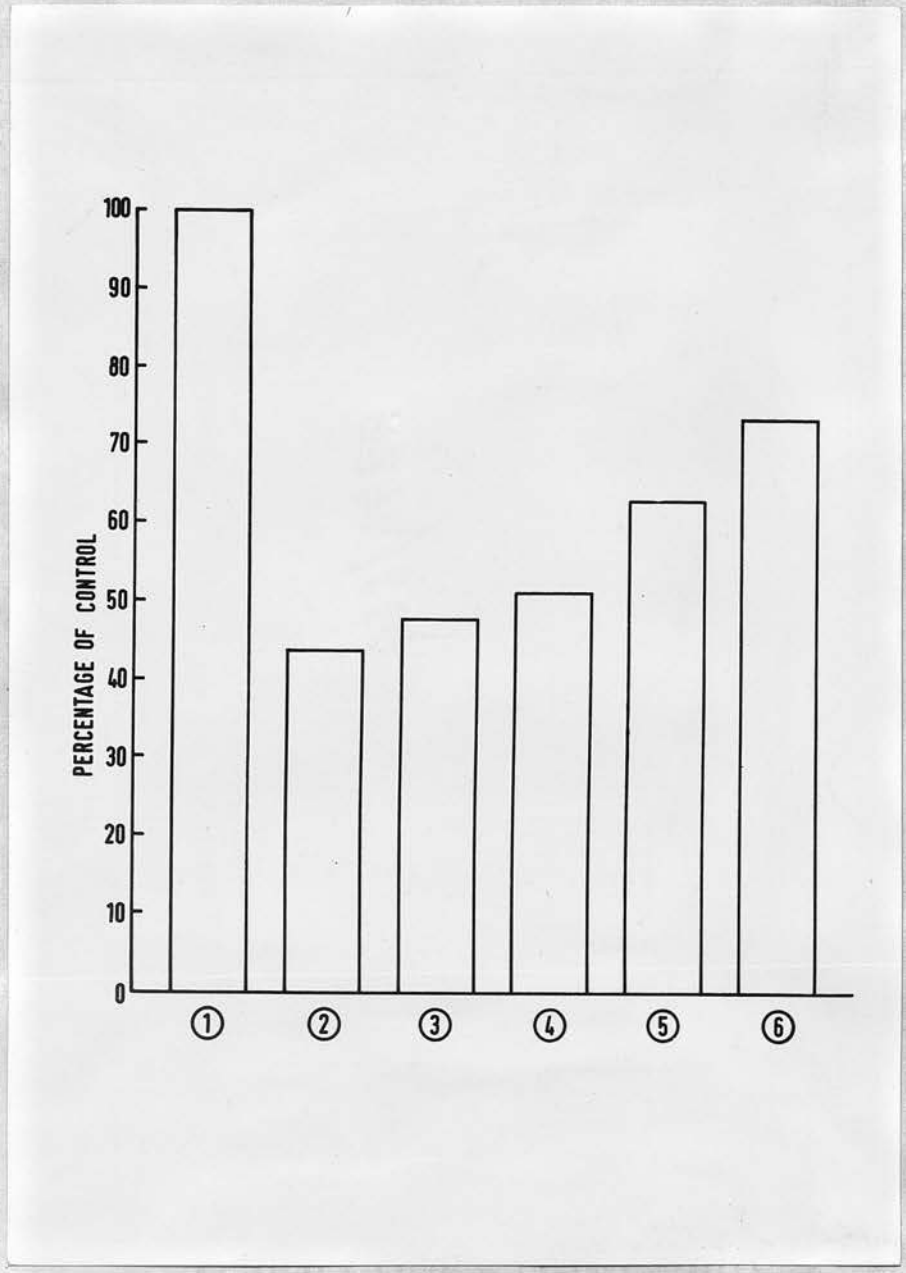


Fig.23. Inhibition of cholesterol side-chain cleavage by various steroids (10µg. per incubation) under standard assay conditions. Enzyme source was supernatant of mitochondrial sonicate.

1. control 2. 20α-hydroxycholesterol 3. 24-hydroxycholesterol
4. 25-hydroxycholesterol 5. 26-hydroxycholesterol
6. pregnenolone.

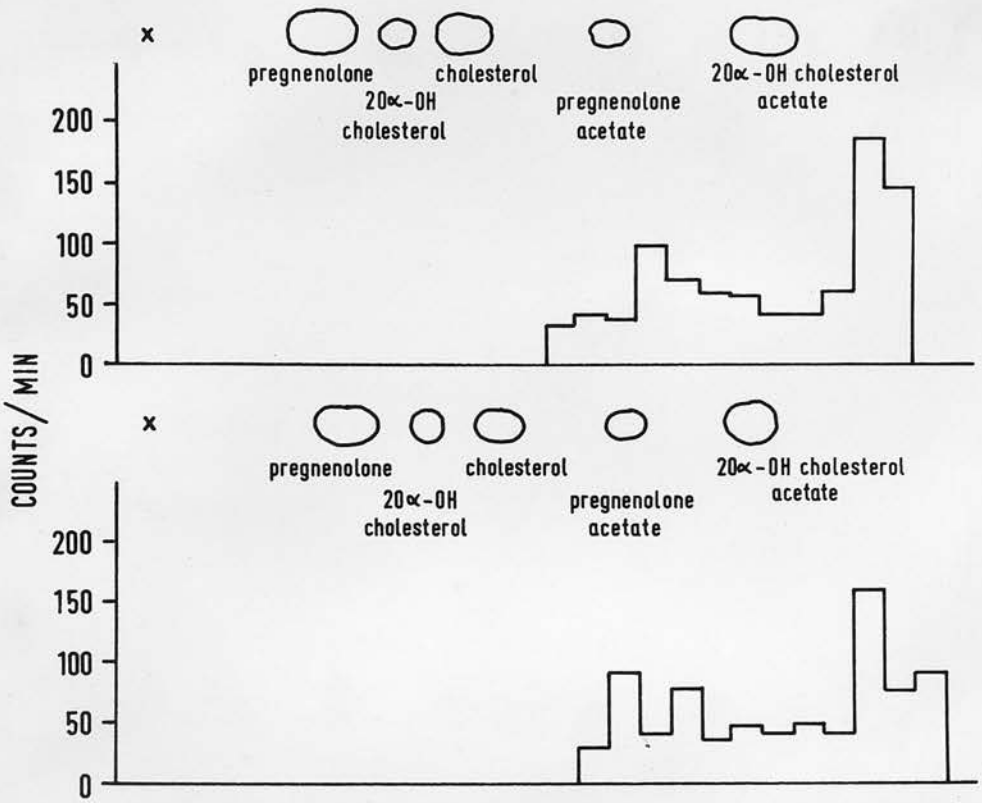


Fig.24. Radiohistograms demonstrating failure to isolate radioactive 20α-hydroxycholesterol-3β-acetate. Solvent system B.

(a) control

(b) in the presence of unlabelled 20α-hydroxy-cholesterol.

(3) Effects of Various Steroids on Side-Chain Cleavage Activity

Hall and Koritz (1964) reported the inhibitory effect of 20α-hydroxycholesterol on the side-chain cleavage of cholesterol. These authors also showed that pregnenolone inhibits this system (Koritz and Hall, 1964). Raggatt and Whitehouse (1966) confirmed these findings.

The effects of various hydroxylated derivatives of cholesterol and pregnenolone on the activity of the side-chain cleavage reaction were studied to see if any intermediates could be accumulated. Fig. 23 shows the results of experiments in which 10 µg. each of 20α-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol, 26-hydroxycholesterol, and pregnenolone were added to incubations. It can be seen that all are inhibitory. The 24-, 25- and 26-hydroxycholesterols were almost as effective inhibitors as 20α-hydroxycholesterol, the effectiveness apparently diminishing as the hydroxyl approached the end of the side-chain. Radiochromatograms of the incubations failed to reveal any accumulated 20α-hydroxycholesterol or 20α,22ξ-dihydroxycholesterol. The region of the thin-layer plate corresponding to the 20α-hydroxycholesterol standard was eluted in the case of the control and the incubation containing 20α-hydroxycholesterol. The material was acetylated with acetic anhydride/pyridine on a hot water bath for 1 hr. along with a few mg. carrier

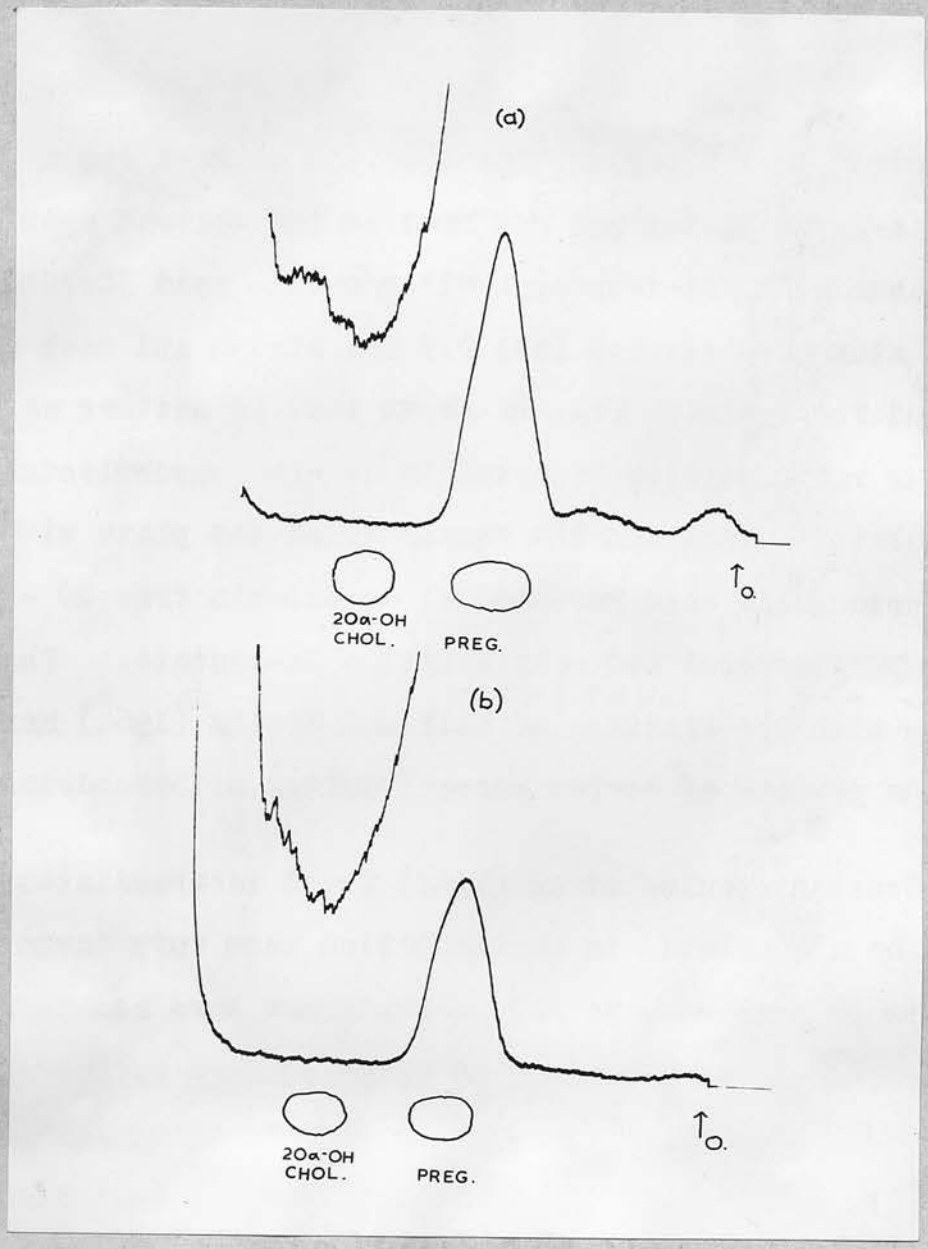


Fig.25. Inhibition of cholesterol side-chain cleavage in the presence of 500μg. pregnenolone. The thin-layer plates were scanned at pulse rate range 100/sec. The region of each plate between cholesterol and pregnenolone was re-scanned at pulse rate range 3/sec. Solvent system B.

(a) control

(b) in the presence of 500μg. pregnenolone.

20α-hydroxycholesterol. The reaction mixture was spotted on thin-layer plates and run once in the solvent system petroleum ether/di-isopropyl ether/acetic acid 30:70:2. The plates were divided into 0.5 cm. strips and each was counted for 1 min. Fig. 24 shows that in neither case was any radioactivity detected in 20α-hydroxycholesterol 3β-acetate. Spraying the remainder of the plate with phosphotungstic acid revealed no detectable free 20α-hydroxycholesterol and only a little di-acetate. This agrees with the findings of Hall and Koritz (1964) using acetone powders of bovine adrenal cortex mitochondria.

Constantopoulos et al (1962) found intermediates could be accumulated in an incubation when very large amounts of pregnenolone and progesterone were added, so an experiment was performed in which 500 μg. pregnenolone was added to an incubation, which was continued for 35 min. This resulted in a 48 per cent inhibition of activity, and greatly suppressed the formation of radioactive progesterone and polar sterols, as would be expected (fig. 25). However, again no accumulated 20α-hydroxy-:cholesterol nor 20α,22ξ-dihydroxycholesterol could be detected, even when the T.L.C. plates were scanned at a low pulse-rate range.

The inhibition of side-chain cleavage activity by 20α-hydroxycholesterol and 25-hydroxycholesterol was studied in greater detail by constructing Lineweaver-Burk

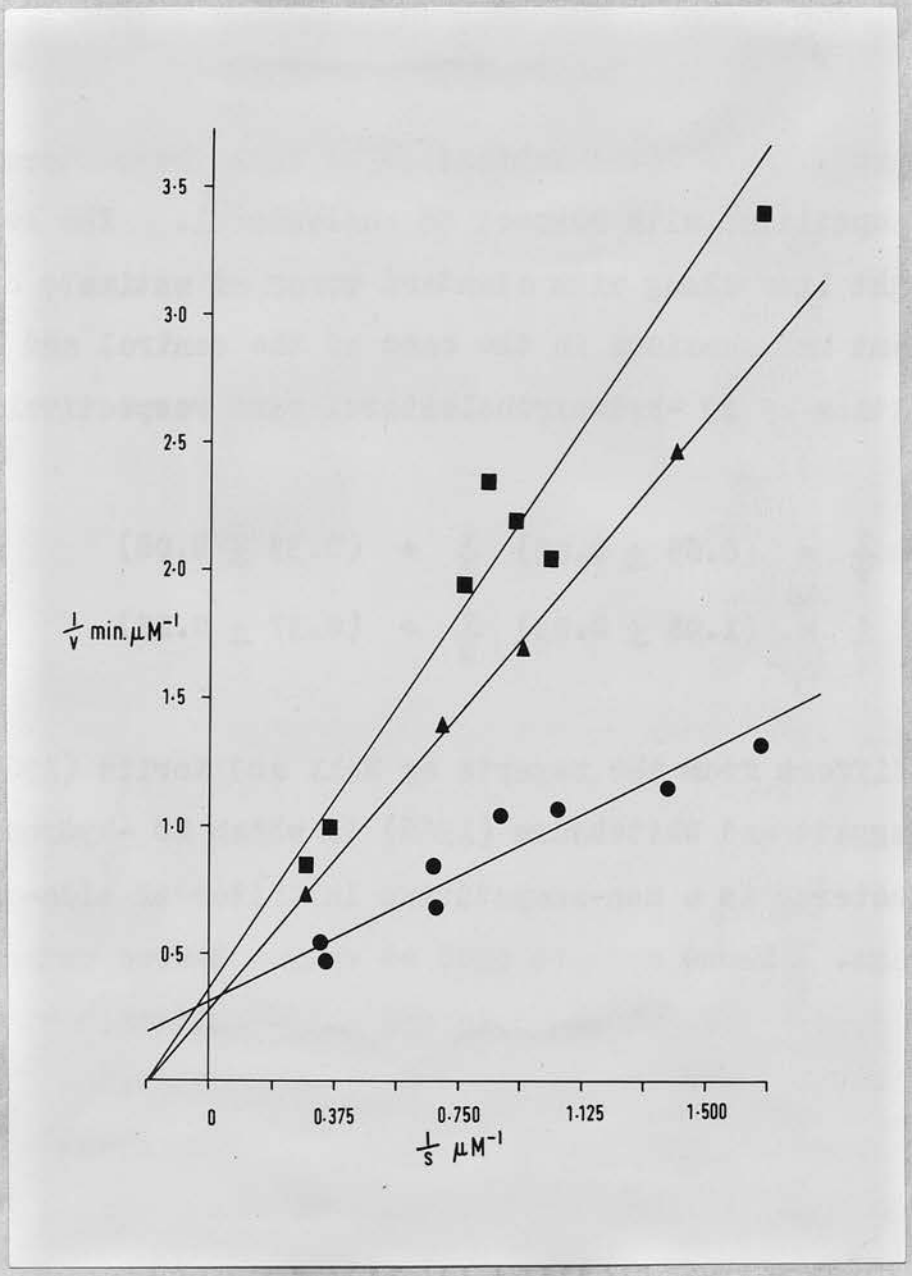


Fig.26. Lineweaver-Burk plots of incubations in the presence of 10μg. each of 20α-hydroxycholesterol and 25-hydroxycholesterol.

v - initial velocity. S - substrate concentration.

● - no addition

▲ - 25-hydroxycholesterol

■ - 20α-hydroxycholesterol

plots as in fig. 26. Inhibition by both these steroids was competitive with respect to cholesterol. The best straight line along with standard error of estimate of gradient and abscissa in the case of the control and inhibition by 20α-hydroxycholesterol were respectively -

$$\frac{1}{v} = (0.65 \pm 0.08)\ \frac{1}{S} + (0.33 \pm 0.08)$$

and, $$\frac{1}{v} = (1.96 \pm 0.23)\ \frac{1}{S} + (0.37 \pm 0.25)$$

This differs from the reports by Hall and Koritz (1964) and Raggatt and Whitehouse (1966) in which 20α-hydroxy-:cholesterol is a non-competitive inhibitor of side-chain cleavage. These authors used as enzyme source extracts of acetone-dried mitochondria and native mitochondria, respectively, whereas in the experiments described here the source of enzyme was a 105,000 xg. supernatant of a mitochondrial sonicate. The value of the apparent Km derived from the data of fig. 26 (1.97 ± 0.53 μM) also differs greatly from that derived by Raggatt and Whitehouse which is 50 μM for whole mitochondria. This could be due to added labelled cholesterol competing with endogenous non-labelled cholesterol in these authors' experiments. A value for the Km of a batch of acetone powder extract was also calculated, and was found to be 4.2 μM.

The most important feature to emerge from these experiments, however, is that the mechanism of the inhibition of the cholesterol side-chain cleavage system by 20α-hydroxycholesterol is not shown to be different from inhibition by other cholesterol derivatives hydroxylated in the side-chain. Thus, we have been unable to obtain any evidence to suggest that the inhibition by 20α-hydroxycholesterol is such as to give it special significance as an intermediate between cholesterol and pregnenolone.

Ability to be metabolised to pregnenolone

The strongest evidence for the involvement of 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol as intermediates in the reaction is therefore the fact that these are more efficient precursors of pregnenolone than is cholesterol. However, the same has been shown to be true for 22-hydroxycholesterol (Chaudhuri, Harada, Shimizu, Gut and Dorfman, 1962), while 20α-hydroxy, 22-ketocholesterol is also metabolised to pregnenolone but has been ruled out as an obligatory intermediate (Constantopoulos, Carpenter, Sato and Tchen, 1966). Thus, if ability to be metabolised to pregnenolone is accepted as evidence for involvement as an intermediate, it is necessary to assume several possible pathways between cholesterol and pregnenolone.

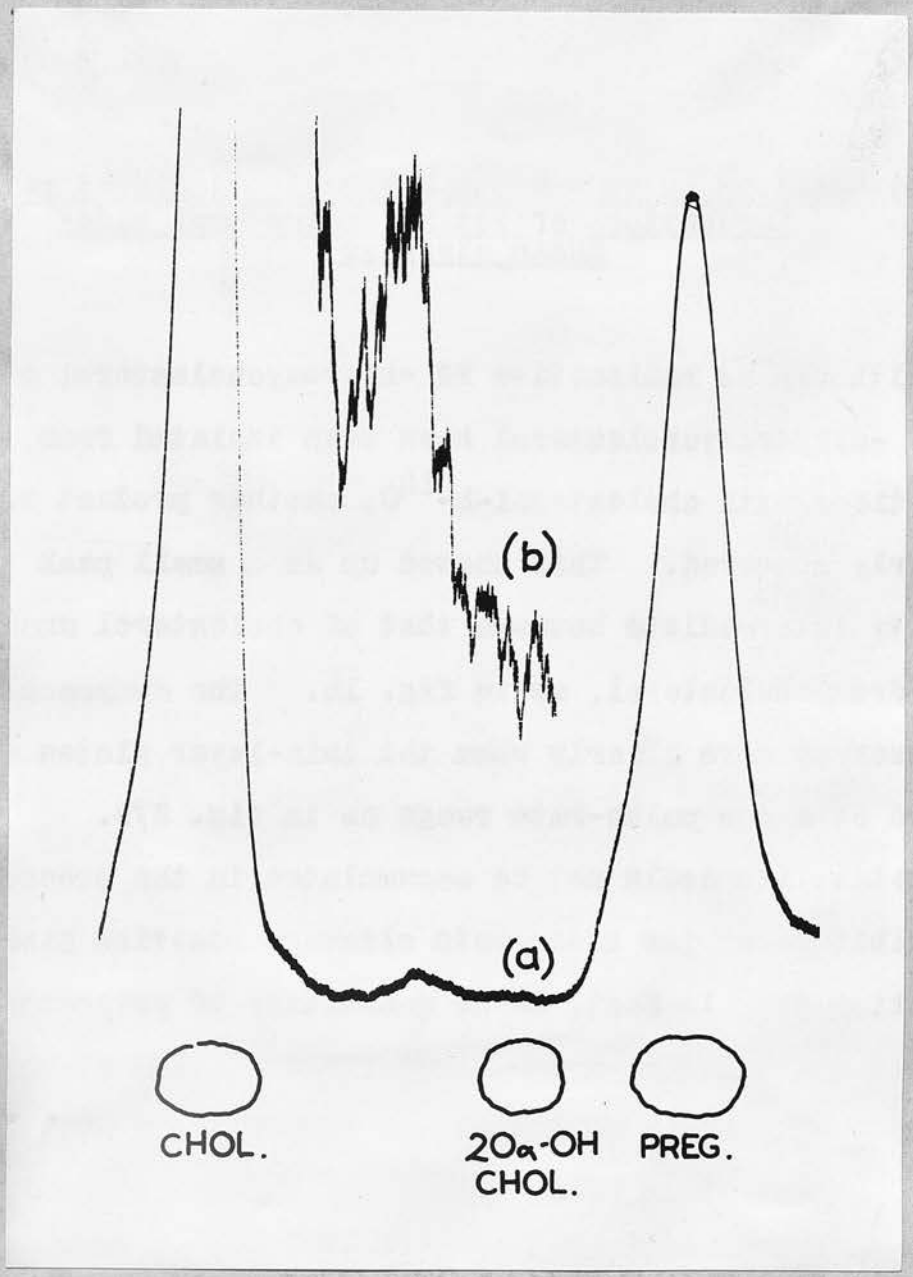


Fig.27. Portion of thin-layer radiochromatogram of a 2hr. incubation of supernatant of mitochondrial sonicate to show unknown metabolite. Solvent system B.

(a) Scanned at pulse rate range 100/sec.

(b) Scanned at pulse rate range 3/sec.

(4) Studies on an unknown Metabolite isolated in incubations effecting cholesterol side-chain cleavage

Although no radioactive 20α-hydroxycholesterol or 20α,22ξ-dihydroxycholesterol have been isolated from incubations with cholesterol-4-^{14}C, another product was regularly observed. This showed up as a small peak of polarity intermediate between that of cholesterol and 20α-hydroxycholesterol, as in fig. 14. The component was observed more clearly when the thin-layer plates were scanned at a low pulse-rate range as in fig. 27b. This metabolite could not be accumulated in the presence of inhibitors of the side-chain cleavage reaction discussed in section 3. In fact, large quantities of pregnenolone added to the incubation inhibited the formation of this material, just as it inhibited the formation of radio-:active pregnenolone (fig. 25). The metabolite did not appear before pregnenolone in a time-course study (fig. 21b). However, it did accumulate when incubation was continued for 2 hr (fig. 27a), although the amount formed relative to that of pregnenolone remained very small. This might suggest that the metabolite is not a precursor of pregnenolone, but rather is formed along with pregnenolone, due perhaps to a side reaction.

Studies on the Structure of the New Metabolite

Studies on the structure of this compound were hampered by the fact that it was present in such small amounts that standard physical-chemical methods of analysis such as infra red spectroscopy and nuclear magnetic resonance spectroscopy would be of no use in tackling the problem. The only way the compound could be detected was by virtue of its radioactivity. Hence the problem was approached by treating the material with various chemical reagents and comparing the polarities of the derivatives formed with those of authentic compounds. In order to acquire sufficient starting material, several incubations were performed for 2 hr. duration, and the region of the T.L.C. plates corresponding to the unknown compound pooled and purified by repeated T.L.C. until a single peak was obtained.

An incubation was performed using cholesterol-26-C^{14} instead of cholesterol-4-C^{14}. This demonstrated that although no material corresponding to pregnenolone or other C-21 steroids was present, the small peak of the unknown metabolite was present, indicating this compound to be a C-27 steroid.

On the basis of the position of the compound on a T.L.C. plate, namely between cholesterol and 20α-hydroxy-:cholesterol, it was speculated that the compound might be

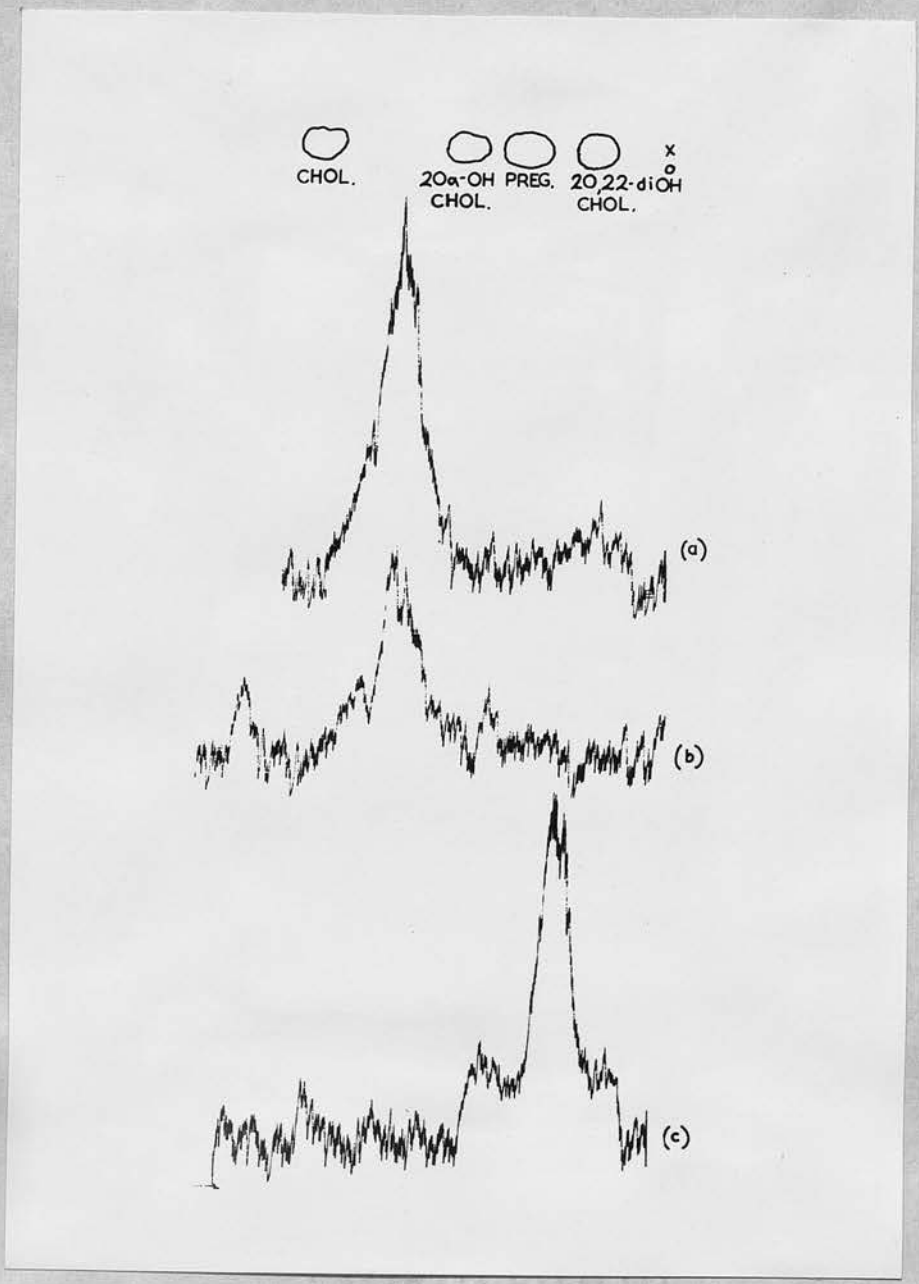


Fig.28. Radiochromatograms of unknown metabolite. Solvent system B.

(a) untreated

(b) treated with potassium iodide in ethanol

(c) treated with lithium aluminium hydride in tetrahydrofuran.

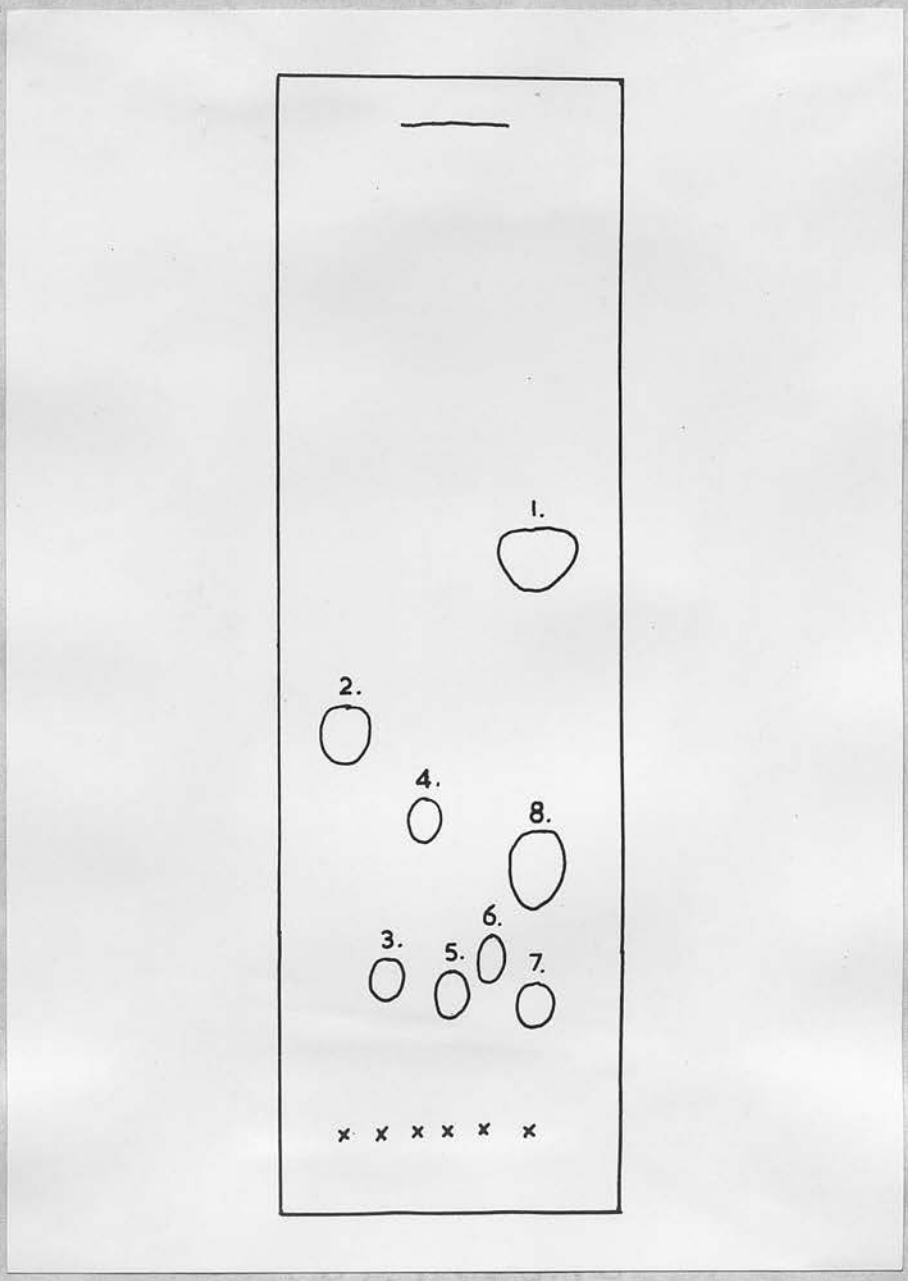


Fig.29. Polarities of cholesterol derivatives hydroxylated in the side-chain in solvent system B.

1. cholesterol 2. 20α-hydroxycholesterol 3. 22-hydroxycholesterol
4. 24-hydroxycholesterol 5. 25-hydroxycholesterol
6. 26-hydroxycholesterol 7. 20α,22R-dihydroxycholesterol
8. pregnenolone.

a cholesterol derivative with an oxygen function in the side-chain such as a ketone, a hydroperoxide, or an epoxide.

Fig. 28a shows a T.L.C. plate of the starting material. When this was left on a plate overnight at room temperature, scraped off and re-run, the polarity did not change, suggesting the material was not decomposed by this treatment.

A sample of the material was left standing for 30 min. at room temperature in ethanol saturated with potassium iodide and containing a few drops of acetic acid. The solvent was evaporated off under nitrogen and the residue spotted on a plate. Fig. 28b shows that this treatment did not affect the polarity. This suggests the compound is not a hydroperoxide.

A further sample of the compound was taken to dryness and dissolved in 1 ml. tetrahydrofuran. A spatula tip of lithium aluminium hydride was added and the mixture refluxed 30 min. on a hot water bath. After filtering, the filtrate was spotted on a T.L.C. plate and run to give fig. 28c. This shows that reduction of the material resulted in an increased polarity, the product running just ahead of 20α,22R-dihydroxycholesterol and overlapping with it. This is suggestive of an epoxide or a ketone being reduced to a hydroxyl. Fig. 29 shows the relative polarities of various cholesterol derivatives

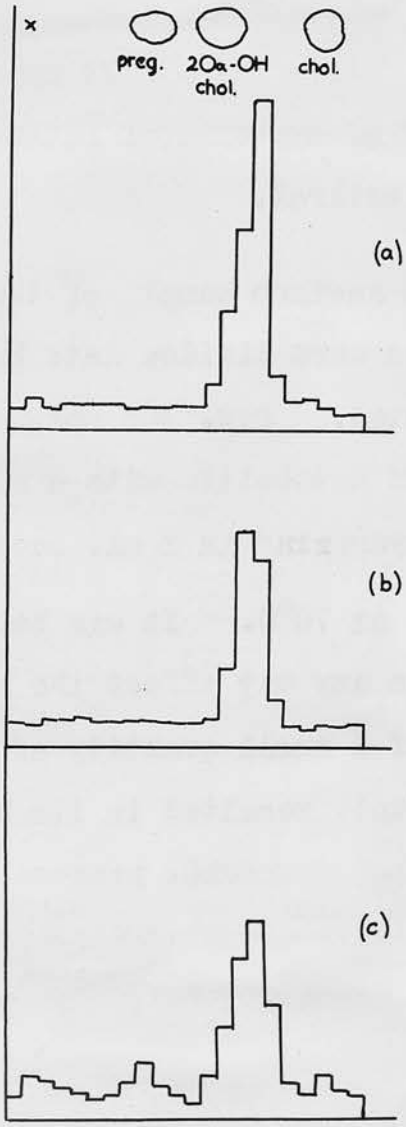


Fig. 30. Radiochromatograms of unknown metabolite. Solvent system B.

(a) untreated

(b) treated with dinitrophenylhydrazine.

(c) incubated with acetone powder extract.

hydroxylated in the side-chain. It appears the compound formed on reduction of the unknown metabolite could have been 22-hydroxycholesterol.

Fig. 30a shows another sample of the unknown metabolite. This time the plates were divided into half-cm. strips and each strip counted. Fig. 30b shows the result of treating the unknown metabolite with a spatula tip of 2,4-dinitrophenyl hydrazine in 2 ml. acetic acid and heating for 15 min. at 70°C. It can be seen that this treatment did not in any way affect the metabolite. Similar treatment of a small quantity of 24-ketonor-:cholesteryl 3β-acetate resulted in its being converted to a less polar yellow compound, presumably the corresponding 2,4-dinitrophenyl hydrazone. It seems unlikely that the unknown metabolite is a ketone.

Fig. 30c shows the result of incubating the unknown metabolite with acetone powder extract under standard conditions for 15 min. There appears to have been a conversion to a compound with the polarity of pregnenolone. Taking the background as 150 counts/5 min., the counts in the region of the pregnenolone standard were 540 counts/5 min., whereas the counts in the unknown were 3880 counts/5 min. This gives a conversion of 12.2 per cent. The conversion of cholesterol to pregnenolone in a parallel incubation was 28.7 per cent.

A further sample of the unknown metabolite was refluxed 30 min. with 10 ml. methanol containing 2 ml. conc. HCl. This treatment did not result in an altered polarity of the material. If the unknown had been an epoxide, this treatment would have been expected to form the chlorhydrin derivative, with a corresponding increase in polarity.

Thus, the nature of the unknown compound is not known. It is a C_{27} sterol, and its polarity suggests it to be a derivative of cholesterol with an oxygen function in the side-chain (oxygen functions in the ring system result in a much larger increase in polarity with respect to cholesterol). Yet it is less polar than 20α-hydroxycholesterol which is itself the least polar of the hydroxycholesterols tested. The substance was reduced to a more polar derivative suggesting the unknown to be either a ketone, a hydroperoxide, or an epoxide, yet evidence for each of these groupings was negative. Although this compound is metabolised to a derivative with the same polarity as pregnenolone, the available evidence suggests that it is a side-product rather than a direct intermediate in the conversion of cholesterol to pregnenolone.

(5) Summary

(a) Attempts have been made to detect proposed intermediates of the cholesterol side-chain cleavage system, namely 20α-hydroxycholesterol and 20α,22ξ-dihydroxy-:cholesterol, in incubations with cholesterol-4-^{14}C.

(b) In a study of the early events in the side-chain cleavage of cholesterol, the first compound detected was pregnenolone, with no evidence of formation either of 20α-hydroxycholesterol or 20α,22ξ-dihydroxycholesterol.

(c) No evidence of any protein-bound intermediate in the cleavage reaction was obtained.

(d) Although 20α-hydroxycholesterol inhibited the cholesterol side-chain cleavage reaction, the same was true of other cholesterol derivatives hydroxylated in the side-chain. Inhibition by these compounds was competitive with respect to cholesterol, and did not lead to any detectable accumulation of 20α-hydroxycholesterol.

(e) A metabolite was detected in incubations with cholesterol-4-^{14}C which did not appear to be an inter-:mediate between cholesterol and pregnenolone, but rather was a side-product of the reaction. Some of the properties of this compound are discussed.

5. STUDIES ON THE EFFECT OF CARBON MONOXIDE ON THE CHOLESTEROL SIDE-CHAIN CLEAVAGE SYSTEM

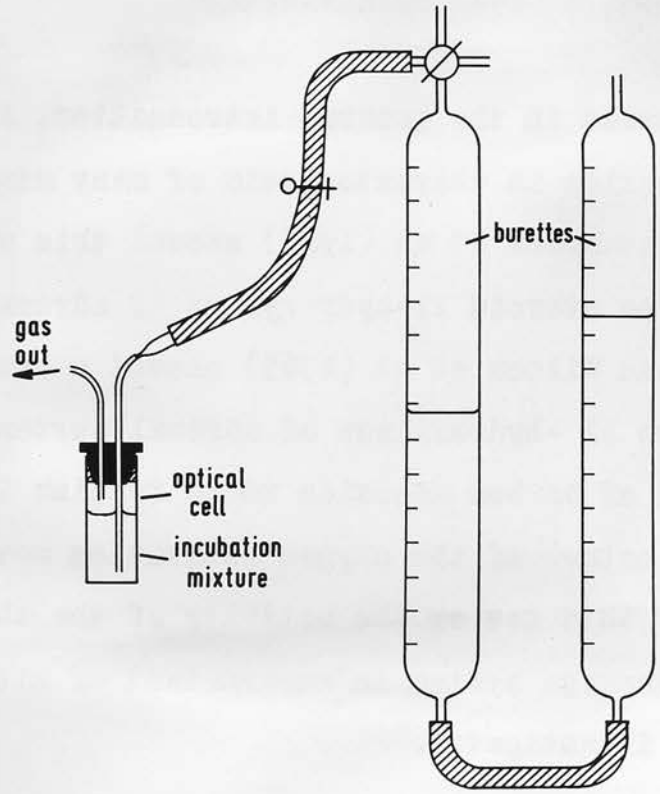


Fig. 31. Apparatus for study of effect of oxygen/carbon monoxide ratio on size of 450mμ chromophore of P-450.

(1) Introduction

As discussed in the general introduction, inhibition by carbon monoxide is characteristic of many mixed-function oxidases. Estabrook et al (1963) showed this was so in the case of the steroid 21-hydroxylase of adrenal cortex microsomes, and Wilson et al (1965) showed a similar effect for the 11β-hydroxylase of adrenal cortex mitochondria. As the effect of carbon monoxide would furnish important clues to the nature of the oxygen activation mechanism, the effect of this gas on the activity of the cholesterol side-chain cleavage system in supernatant of mitochondrial sonicate was investigated.

Fig. 31 shows the apparatus used. Carbon monoxide was prepared by the action of conc. sulphuric acid on formic acid (both analar grade). The gas was bubbled through water to remove acid vapours, then through chromous chloride solution to remove oxygen, and finally through potassium hydroxide pellets to dry it. Nitrogen was also bubbled through chromous chloride solution to ensure it was oxygen free. Appropriate gas mixtures were prepared in the 1 litre burettes. Incubations were performed in 3 ml. optical cuvettes. The cuvettes were fitted with Teflon caps drilled to permit insertion of Portex vinyl tubing. Gas mixtures were bubbled through the cells by maintaining a constant head of

pressure. A steady stream of bubbles was maintained throughout to ensure continuous agitation. Thus, this gas system is an open one, in contrast to the closed system of Warburg (1949) and Estabrook et al (1963) in which the incubation flask was connected to a Warburg manometer. This system was chosen because it utilised equipment available in the laboratory, and also because the same apparatus could be used for optical studies and for incubations with cholesterol-4-^{14}C.

(2) Effect of Carbon Monoxide on the size of the P-450 chromophore

These studies were performed at room temperature because the 'Optica' spectrophotometer did not have a water-jacketed cell holder. The volume of incubation mixture shown in Table 2 was made up, minus the cholesterol-4-^{14}C, and after standing a few minutes to reach equilibrium with respect to temperature and state of reduction of the pyridine nucleotides, the mixture was divided equally between two optical cuvettes. One was placed in the reference light path, and the other in the experimental light path, of the spectrophotometer. The region of the spectrum between 400 mμ and 480 mμ was scanned to give a base line, and then the experimental cell was gassed with the appropriate gas mixture for 4 min., and the difference spectrum recorded. At first, both cells were gassed with nitrogen to give the base-line, but it was found that there was no detectable difference between the carbon monoxide-nitrogen and the carbon monoxide-air difference spectra. Fig. 18 shows such a difference spectrum when the experimental cuvette was gassed with 100 per cent carbon monoxide. It can be seen that the chromophore at 450 mμ, characteristic of cytochrome P-450, was rather small. This made accurate determinations of small changes in the size of this chromophore somewhat difficult. The extinction at 450 mμ minus the extinction at 480 mμ was taken as the

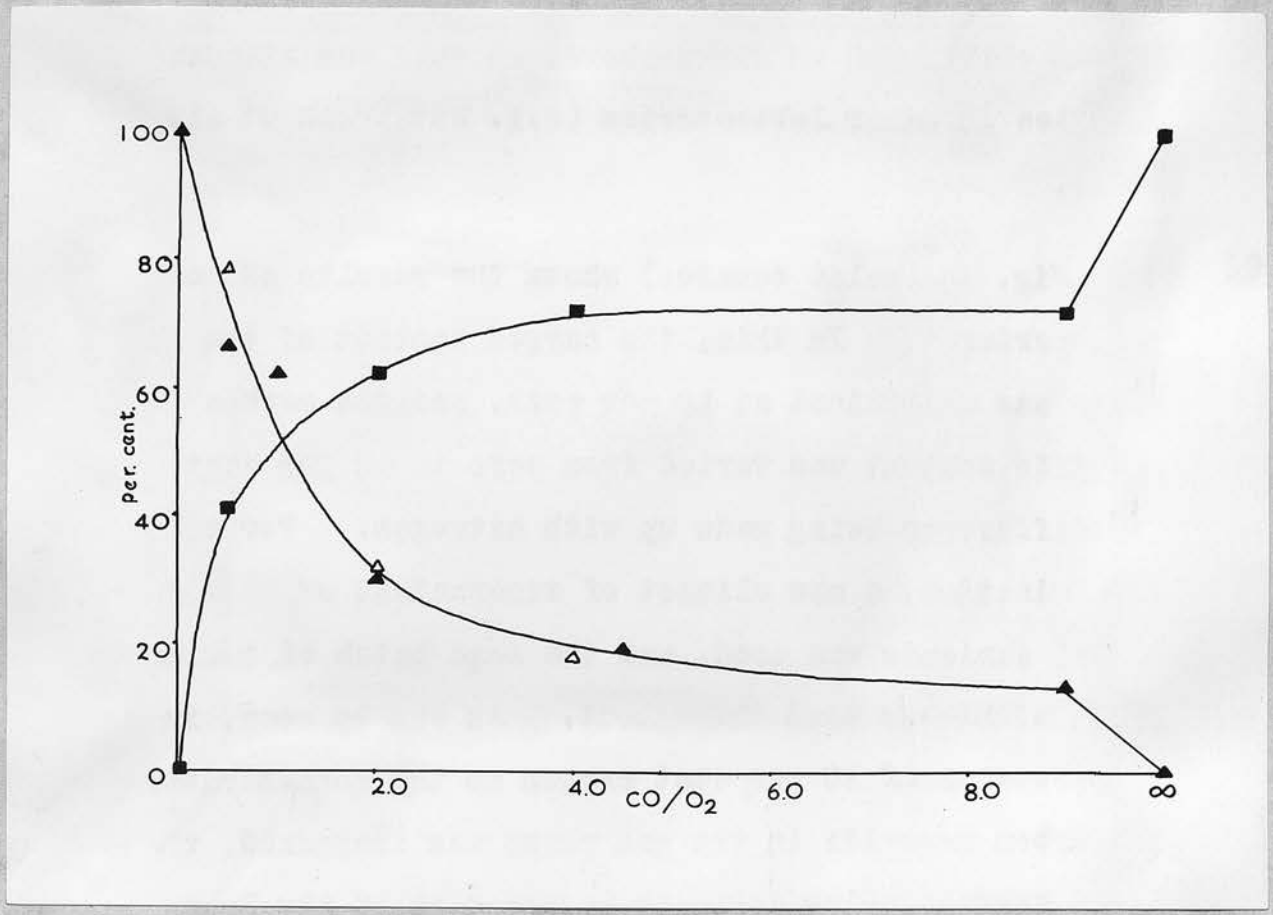


Fig. 32. Effect of oxygen/carbon monoxide ratio on rate of cholesterol side-chain cleavage and size of P-450 chromophore.

■ - height of 450mμ chromophore (percentage of height in the presence of 100% carbon monoxide.)

▲ - rate of side-chain cleavage (percentage of rate in the absence of carbon monoxide.) Oxygen content of gas phase - 10%.

△ - rate of side-chain cleavage (percentage of rate in the absence of carbon monoxide.) Oxygen content of gas phase - 20%.

height of the 450 mμ chromophore, as this was standard practice in other laboratories (e.g. Estabrook et al, 1963).

Fig. 32 (solid squares) shows the results of such an experiment. In this, the oxygen content of the gas phase was maintained at 10 per cent, and the carbon monoxide content was varied from zero to 90 per cent, the difference being made up with nitrogen. For each determination, a new aliquot of supernatant of mitochon-:drial sonicate was used, and the same batch of sonicate supernatant was used throughout. As can be seen, in the presence of 10 per cent oxygen as the concentration of carbon monoxide in the gas phase was increased, there was a corresponding increase in the size of the P-450 chromophore until saturation was reached at around 40 per cent carbon monoxide. Further increase in the carbon monoxide content did not greatly affect the height of the chromophore. In the presence of 100 per cent carbon monoxide, there was a further increase in the height of the chromophore. That the kinetics of formation of the 450 mμ chromophore appear to be consistent with a competition between carbon monoxide and oxygen will be discussed in section 4.

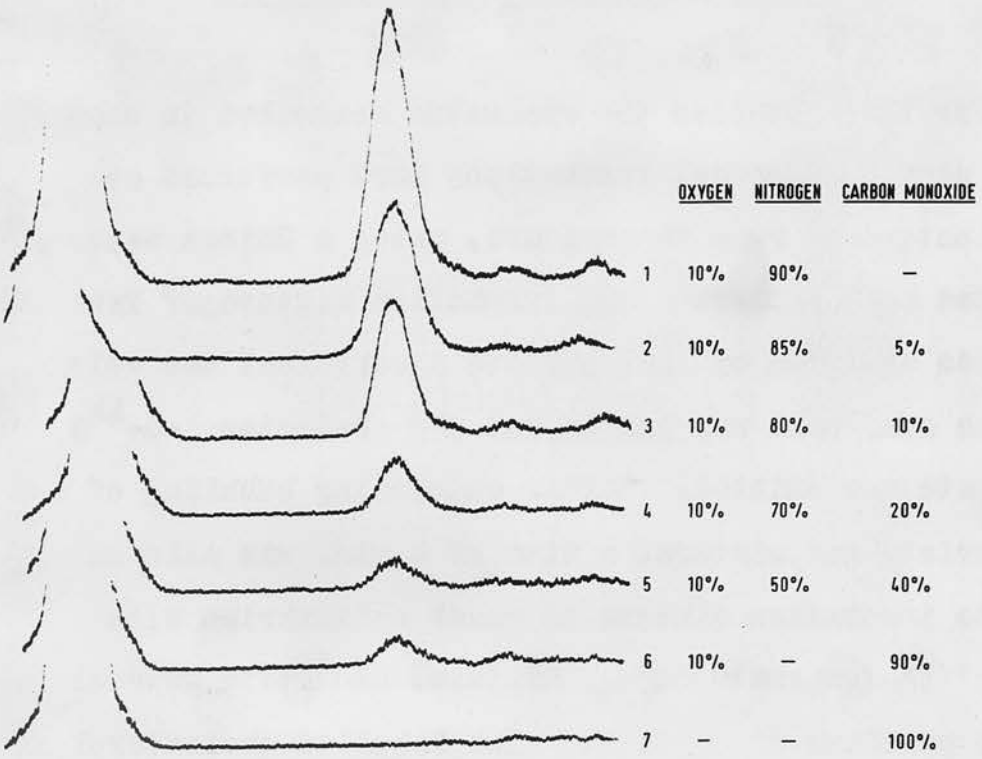


Fig. 33. Thin-layer radiochromatograms of incubations performed under the conditions shown in the accompanying table. Solvent system A.

(3) Effect of Carbon Monoxide on the activity of the side-chain cleavage system

For these studies the apparatus described in section 1 was used. However, incubations were performed at 37°C instead of room temperature, using a Unicam water-jacketed cell holder. The incubation mixture of Table 2 was made up in an optical cuvette except that the volume of each component was halved and the cholesterol-4-^{14}C substrate was omitted. After commencing bubbling of the appropriate gas mixture, a time of 4 min. was allowed for the incubation mixture to reach equilibrium with respect to temperature, gas mixture, and state of reduction of the pyridine nucleotide. The labelled cholesterol substrate was then added from an Agla syringe by removing the outlet tubing and inserting the syringe in the hole in the Teflon cap. The outlet tubing was replaced immediately. The bubbling of the gas through the cell ensured rapid mixing of the substrate. The incubation was allowed to proceed for 15 min. and then stopped with methanol and extracted in the usual way. The presence of the outlet tube was found to be essential. If it was absent, sufficient oxygen was apparently able to enter the cell to alter the oxygen/carbon monoxide ratio and cause erroneous results.

Fig. 33 shows a series of radiochromatograms of incubations performed under the conditions shown in the accompanying table. The numerical results are shown in

Table 6

Inhibition of cholesterol side-chain cleavage by carbon monoxide

Percentage carbon monoxide		Percentage conversion to pregnenolone (control=100%)
10% O_2	20% O_2	
0		100
5		66
10		62
20		30
45		19.5
90		13.5
	10	78
	20	62
	80	18.5

conversion in the presence of 100% carbon monoxide = zero

Table 6. Also in Table 6 are some determinations made when the gas phase contained 20 per cent oxygen. A graphical plot of the results of Table 6 is shown in fig. 32 (triangles). Broadly speaking, the results for the effect of carbon monoxide on the enzymic activity were the mirror image of the results for the effect on the size of the 450 mμ chromophore. As the carbon monoxide content of the gas phase increased, there was a corresponding decrease in the activity of the cholesterol side-chain cleavage system, until a state of saturation was approached, at an oxygen to carbon monoxide ratio of about 1:4. Further increase in the carbon monoxide content did not cause a great decrease in enzymic activity. In the presence of 100 per cent carbon monoxide, there was no activity. Irrespective of the concentration of oxygen in the gas phase, all the points fell on the same line; that is to say, within the range of oxygen concentration used in this experiment, the enzymic activity depended only on the ratio of oxygen to carbon monoxide.

Again, the kinetics of the inhibition are consistent with the theory developed in section 4.

(4) Calculation of the Partition Constant

Warburg (1949) derived the following expression to account for the inhibition by carbon monoxide of cellular respiration. In the case of the cholesterol side-chain cleavage system, it is assumed that carbon monoxide is competing with oxygen for an enzyme site resulting in inhibition of side-chain cleavage activity and appearance of an absorption maximum at 450 mμ in the reduced differ-:ence spectrum.

For the reaction -

$$E.O_2 + CO \rightleftharpoons E.CO + O_2$$

where E is the binding site, we have the equilibrium constant -

$$K = \frac{(E.O_2)(CO)}{(E.CO)(O_2)}$$

therefore -

$$\frac{CO}{O_2} = K. \frac{E.CO}{E.O_2}$$

hence -

$$\frac{CO}{O_2} = K. \frac{1 - \frac{r_{co}}{r_o}}{\frac{r_{co}}{r_o}} \qquad \text{(1)}$$

Where r_{co} is the reaction rate in the presence of carbon monoxide and r_o the reaction rate when no carbon monoxide

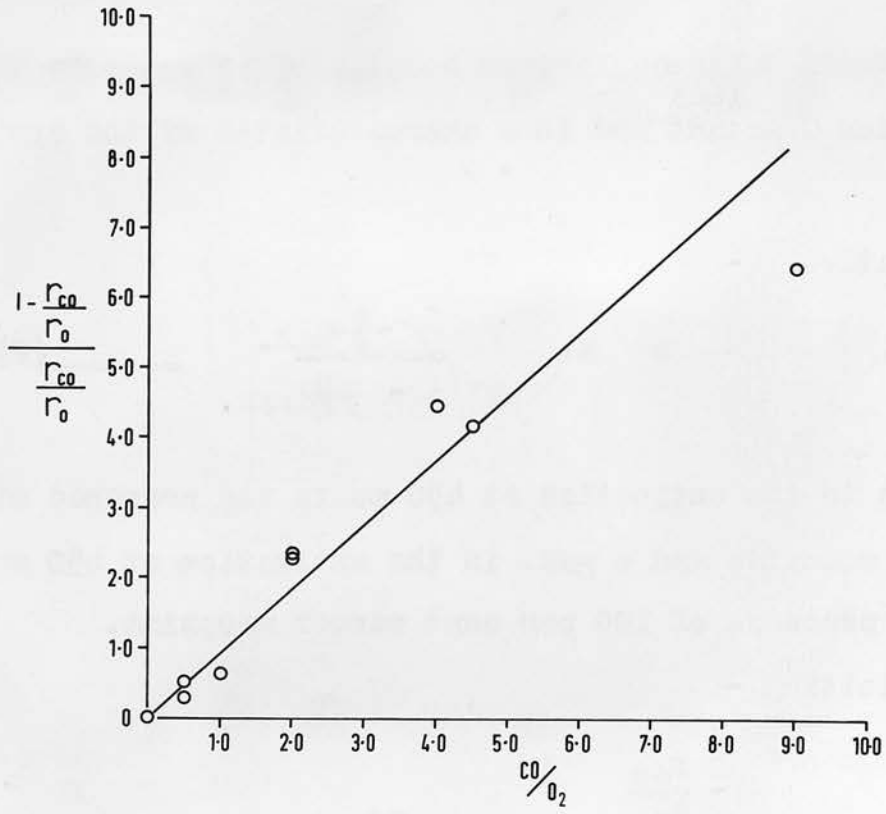


Fig. 34. Plot of $\frac{1 - \frac{r_{co}}{r_o}}{\frac{r_{co}}{r_o}}$ versus $\frac{CO}{O_2}$ for

cholesterol side-chain cleavage. The gradient is

$\frac{1}{K}$

is present. The equilibrium constant K is known as the Partition Constant and is a characteristic of the system.

Alternatively -

$$\frac{CO}{O_2} = K. \frac{\frac{e}{e\ max.}}{1 - \frac{e}{e\ max.}} \qquad (2)$$

Where e is the extinction at 450 mμ in the presence of carbon monoxide and e max. is the extinction at 450 mμ in the presence of 100 per cent carbon monoxide.
Thus plotting -

$$\frac{1 - \frac{r_{co}}{r_o}}{\frac{r_{co}}{r_o}} \text{ versus } \frac{CO}{O_2}$$

or,

$$\frac{\frac{e}{e\ max.}}{1 - \frac{e}{e\ max.}} \text{ versus } \frac{CO}{O_2}$$

should give a straight line of gradient 1/K.

When the data of Table 6 was plotted in this way, fig. 34 was obtained. As can be seen, the points are a good fit to a straight line. The line drawn through the points is the regression line of -

$$\frac{1 - \frac{r_{co}}{r_o}}{\frac{r_{co}}{r_o}} \text{ on } \frac{CO}{O_2}$$

calculated on the assumption that the line passes through

the origin. From this the value of K and standard error of estimate is - K = 1.22 ± 0.13.

In fact, this is the highest value for the partition constant we have obtained. Using different batches of adrenals, the value of the partition constant has been found to vary between 0.2 and 1.2. The reason for this variation is not known, but it was observed that the partition constant was generally lower in preparations which were fresh than in preparations which had been stored for a week or two in the deep freeze. This finding is in keeping with those of Estabrook et al (1963) who quote values between 0.6 and 1.8 for the partition constant of the steroid 21-hydroxylase of adrenal cortex microsomes.

The partition constant was also calculated for the variation in height of the 450 mμ chromophore with the oxygen/carbon monoxide ratio. The value calculated in this way from the data of three separate experiments was - K = 1.07 ± 0.30.

Thus, in common with several other mixed-function oxidases, and more particularly the steroid 11β- and 21-hydroxylases, the cholesterol side-chain cleavage system is inhibited by carbon monoxide. Furthermore, the kinetics of this inhibition are consistent with a competition between oxygen and carbon monoxide for a

common binding site. Such competition is a well known feature of several haemoproteins such as cytochrome oxidase and haemoglobin. The partition constant for the cholesterol side-chain cleavage system, although somewhat variable from one preparation to another, is about unity, which is the same as published values for the steroid 21-hydroxylase. This value of the partition constant for mixed-function oxidases is quite different from the partition constants of haemoglobin and cytochrome oxidase which are $1.8 - 8.0 \times 10^{-3}$ and 10 respectively (Keilin and Wang, 1946). It is, however, similar to the partition constant of cytochrome P-450, which was calculated here to be 1.07 ± 0.30. This suggests the possibility that cytochrome P-450 may be involved as the oxygen-binding site for the cholesterol side-chain cleavage system.

(5) Summary

(a) Preparations which contain cholesterol side-chain cleavage activity also contain a chromophore in the reduced carbon monoxide difference spectrum characteristic of cytochrome P-450.

(b) The cholesterol side-chain cleavage system is inhibited by carbon monoxide, this inhibition depending only on the ratio of carbon monoxide to oxygen in the gas phase.

(c) The kinetics of this inhibition are suggestive of a competition between carbon monoxide and oxygen for a common binding site. The partition constant for this competition varies from 0.2 - 1.2.

(d) This value for the partition constant of the cholesterol side-chain cleavage system is similar to that for the steroid 21-hydroxylase.

(e) The value for the partition constant of cyto-:chrome P-450 is also about unity and is quite different from that of other haemoproteins such as cytochrome oxidase and haemoglobin.

(f) These findings are consistent with the hypothesis that cytochrome P-450 is involved in oxygen binding in the cholesterol side-chain cleavage system.

6. THE EFFECT OF LIGHT ON THE CARBON MONOXIDE INHIBITION OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE SYSTEM

(1) Introduction

A characteristic of the carbon monoxide complexes of haemoproteins is that the combination can be reversed by light. Estabrook et al (1963) and Omura et al (1965) showed that the carbon monoxide inhibition of mixed-function oxidases, and particularly the steroid 21-hydroxylase was light-reversible and furthermore the most effective wavelength for reversing the inhibition was 450 mμ. This greatly strengthened the case for the involvement of cytochrome P-450 as the site of oxygen-binding in these mixed-function oxidases. It was necessary therefore to examine the carbon monoxide inhibition of the cholesterol side-chain cleavage system for its light-reversibility.

Theory

According to Warburg (1949), the partition constant without light is -

$$K_d = \left(\frac{\frac{r_{co}}{r_o}}{1 - \frac{r_{co}}{r_o}} \right)_d \cdot \frac{CO}{O_2}$$

where subscript d refers to reactions in the absence of light.

On exposure to light we have a new constant -

$$K_1 = \left(\frac{\frac{r_{co}}{r_o}}{1 - \frac{r_{co}}{r_o}} \right)_1 \cdot \frac{CO}{O_2}$$

where subscript 1 refers to reactions in the presence of light.

The percentage displacement of the partition constant -

$$\frac{\Delta K}{K_d} = \frac{K_1 - K_d}{K_d}$$

is proportioned within limits to the light intensity i for any wavelength.

therefore -

$$\frac{\Delta K}{K_d} = Li$$

where L is a proportionality factor called "light sensitivity" whose dimensions are reciprocal of intensity. Hence plotting L against the wavelength of the incident light will give the photochemical action spectrum of the carbon monoxide-sensitive system, and will reveal which wavelength is the most effective in reversing the inhibition.

Experimental Procedure

For their studies on the photochemical action spectrum of the steroid 21-hydroxylase, Omura et al (1965) used a 1.6 K.watt high pressure Xenon lamp, the light from

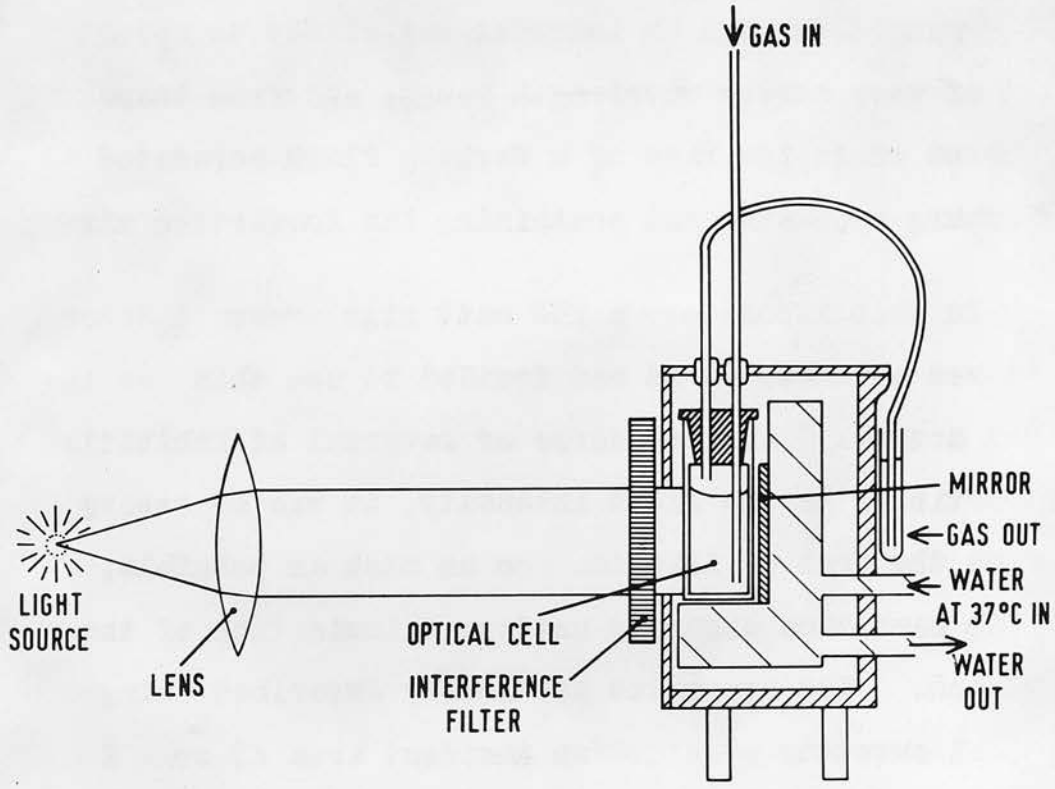


Fig. 35. Apparatus for study of light reversal of carbon monoxide inhibition of cholesterol side-chain cleavage.

which passed through an interference filter to select a band of very narrow wavelength range, and from thence was directed on to the base of a Warburg flask connected to a Warburg manometer and containing the incubation mixture.

In this laboratory a 250 watt high pressure Xenon lamp was at hand, so it was decided to use this as the light source. As the degree of reversal of inhibition is proportional to the light intensity, it was necessary to reduce the area of illumination as much as possible, while at the same time ensuring uniform illumination of the entire solution. The apparatus previously described using the optical cuvettes provided an incident area of some 2 $cm.^2$ A mirror was placed behind the cuvette, serving to almost double the light flux. The experimental arrangement is shown in fig. 35. The cuvette was kept at 37°C in the Unicam water-jacketed cell holder. The lamp was enclosed in an aluminium shielding blackened on the inside, save for a slit. The lens was arranged to cause the light beam leaving the slit to focus on the area of the incub-:ation mixture.

The filters used were interference filters (Grubb Parsons Ltd., Newcastle-upon-Tyne). The average band width was 8-10 mμ. Nine interference filters were used with transmission maxima at 403, 410, 418, 429, 448, 456, 470, 491 and 502 mμ. The relative light intensity of the lamp beam passing through each filter was determined using

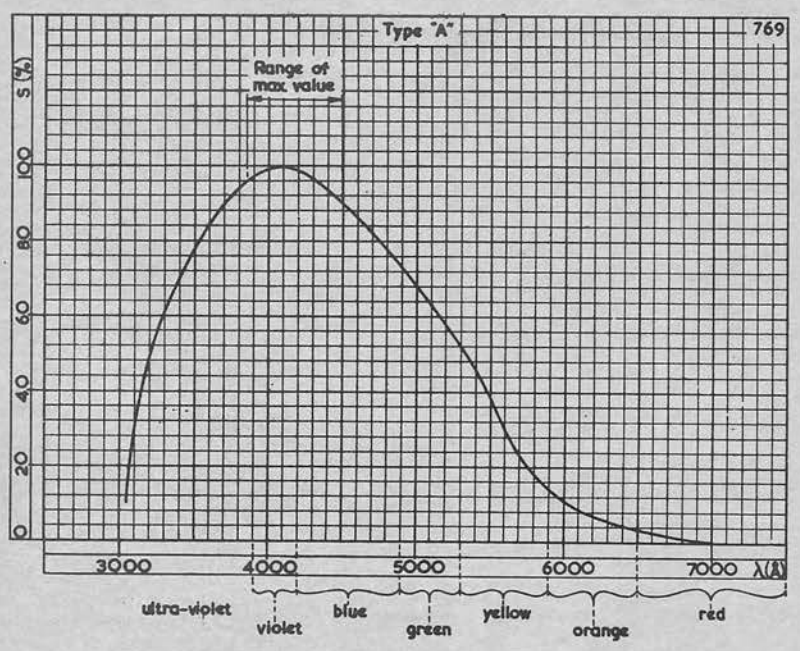


Fig. 36. Spectral response of Mullard photocathode type "A" (caesium-antimony)

Table 7

Relative intensity of light at each wavelength

wavelength of maximum transmission of filter(mμ)	photocell reading	spectral response	relative light intensity (450mμ=1)
403	5.50	0.990	0.82
410	5.15	1.000	0.76
418	3.37	0.985	0.57
429	5.93	0.960	0.91
448	6.10	0.900	1.00
456	4.45	0.870	0.76
470	7.92	0.810	1.44
491	4.65	0.730	0.94
502	2.20	0.680	0.48

a Mullard photo-emissive cell with a type 'A' caesium-antimony photocathode, connected to a Varian 33B-2 electrometer. Fig. 36 shows the spectral response curve of this cell. Two layers of fine-mesh steel gauze were placed across the photocell aperture to act as a neutral density filter to limit the amount of light striking the photocathode to measureable proportions. Table 7 shows the relative light intensities for each wavelength used.

The experimental procedure was exactly as described for experiments on the carbon monoxide inhibition. The enzyme preparation used was once again supernatant of mitochondrial sonicate. The gas outlet tubing was placed in a small tube of water in order to observe whether or not the gas was continuing to bubble through the incubation mixture throughout the incubation. During the 4 min. pre-incubation, light passing through the appropriate filter illuminated the incubation mixture.

(2) Effect of light on the Carbon Monoxide Inhibition

The first experiment was designed to find out whether or not the whole light beam from the lamp, that is, without any filters present, was effective in reversing the carbon monoxide inhibition.

Incubations were performed under the following gas phases:-

1	2 and 3	4 and 5
10% O_2	10% O_2	10% O_2
90% N_2	70% N_2	80% N_2
	20% CO	10% CO

Incubations 1, 2 and 4 were conducted in the dark, and incubations 3 and 5 were conducted with the cell illuminated. The results were:-

Incubation	Percentage conversion
1	12.1
2	3.6
3	6.8
4	5.6
5	9.2

This shows clearly that the light beam was capable of reversing the carbon monoxide inhibition. This was not a thermal effect, as the light beam caused no heating of the cell when the gas was bubbling through the incubation mixture, although a rise of a few degrees was observed when no gas was bubbling through the mixture. It was

decided to adopt a gas phase consisting of 10 per cent oxygen, 80 per cent nitrogen, and 10 per cent carbon monoxide for subsequent investigations, because, as the value of the partition constant is about unity, the ratio $\frac{(E.CO)}{(E.O_2)}$ will also be about unity at this carbon monoxide/oxygen ratio. Hence the enzymic activity should be most sensitive to light with a gas mixture of this composition.

(3) The photochemical action spectrum of the cholesterol side-chain cleavage system - Initial Experiments

For the initial experiments, five interference filters were used, with transmission maxima at 410, 429, 448, 470 and 491 mμ. The gas phase was 10 per cent oxygen, 80 per cent nitrogen and 10 per cent carbon monoxide throughout. Five values were determined for each wavelength, three values for one batch of sonicate supernatant, and two values for a second batch. In each experiment, one value for each filter was determined, along with the two controls, namely the incubation performed in the presence of carbon monoxide but in the absence of light, and the incubation performed in the absence of carbon monoxide. To calculate ΔK, the average value of K_d for the particular batch of sonicate supernatant was subtracted from the value of K_1 in each case. The final results were expressed as the ratio -

$$\frac{L}{L_{450}} = \frac{i_{450} \cdot \frac{\Delta K}{K_d}}{i \cdot \frac{\Delta K_{450}}{K_d}}$$

where subscript 450 refers to the value at 450 mμ. This ratio was calculated from the mean value of ΔK for each wavelength. The variance of the expression was calculated from the formula -

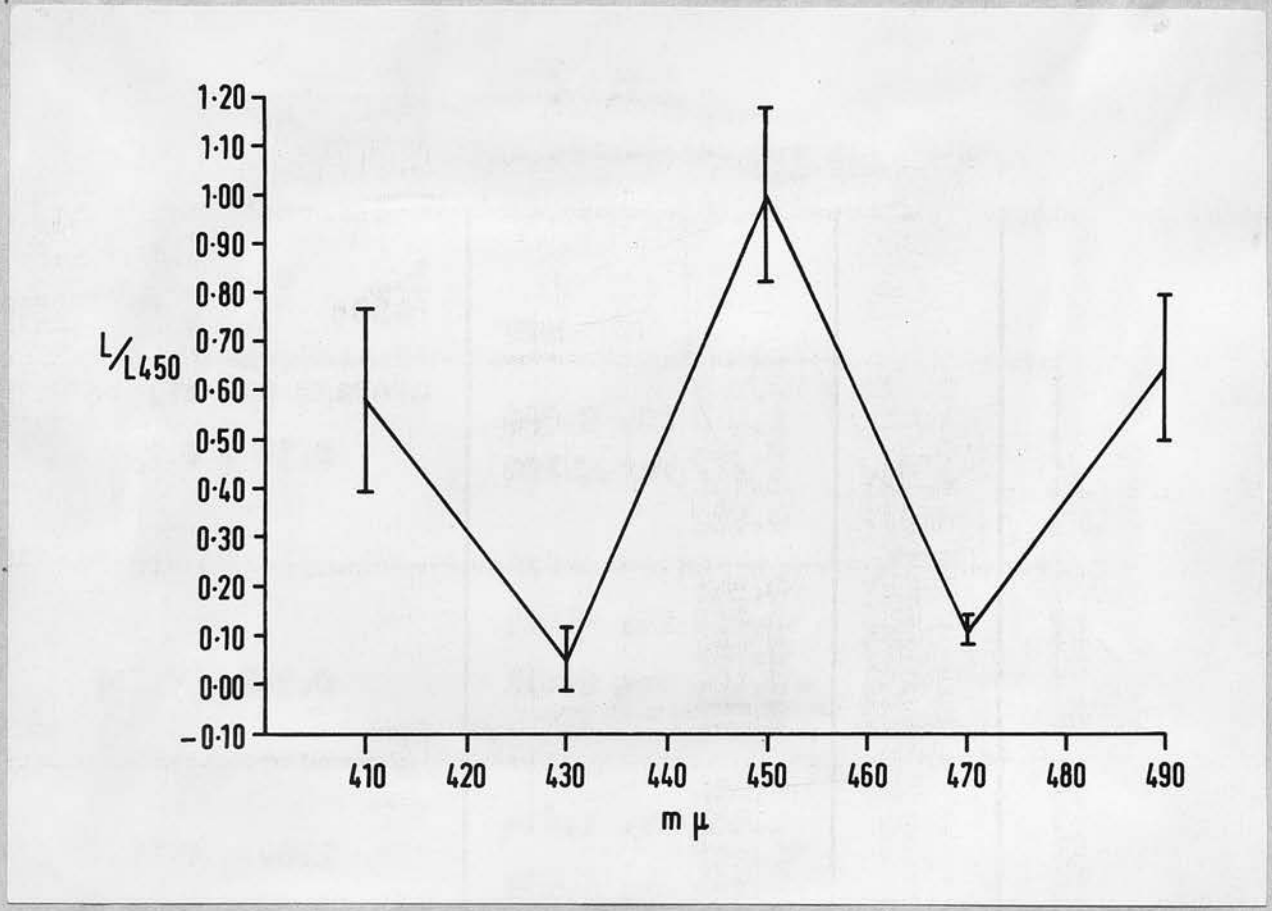


Fig. 37. Photochemical action spectrum for light-reversal of carbon monoxide inhibition of cholesterol side-chain cleavage. (preliminary experiments)

Table 8

Data for 1st photochem. action spectrum

Filter	$\frac{r_{co}}{r_o}$	K_1	ΔK	average and variance	$\frac{L}{L_{450}} = \frac{i_{450}}{i} \frac{\Delta \frac{K}{K_d}}{\Delta \frac{K_{450}}{K_d}}$
410 mμ	0.412	0.700	0.508		average and standard error
	0.621	1.64	1.448	av. 0.666	
	0.353	0.556	0.364		0.58 ± 0.19
	0.424	0.737	0.391	var. 0.200	
	0.492	0.967	0.621		
429 mμ	0.353	0.547	0.345		
	0.192	0.239	0.037	av. 0.064	
	0.229	0.297	0.105		
	0.135	0.156	-0.190	var. 0.031	0.048 ± 0.053
	0.268	0.367	0.021		
448 mμ	0.723	2.82	2.628		
	0.643	1.80	1.608	av. 1.614	
	0.643	1.80	1.608		1.00 ± 0.19
	0.620	1.63	1.284	var. 0.400	
	0.581	1.39	0.944		
470 mμ	0.211	0.267	0.075		
	0.169	0.204	0.012	av. 0.241	0.11 ± 0.03
	0.480	0.924	0.732		
	0.416	0.711	0.365	var. 0.110	
	0.269	0.368	0.022		
491 mμ	0.562	1.28	1.088		
	0.467	0.875	0.683	av. 0.883	0.65 ± 0.15
	0.602	1.51	1.164	var. 0.08	
	0.485	0.943	0.597		

K_d for first three determinations at each wavelength 0.192

K_d for second two determinations at each wavelength 0.346

$$\frac{V_x}{x^2} = \frac{V_y}{y^2} + \frac{V_z}{z^2}$$

where $x = \frac{y}{z}$ and V = variance.

The spread of results was expressed as the standard error of the estimate.

Table 8 shows the data obtained. The resulting photochemical action spectrum is shown in fig. 37. As can be seen, 448 mμ was the wavelength most effective for reversing the carbon monoxide inhibition. However, 410 mμ and 491 mμ were also effective in reversal of inhibition. Study of the data shows that this effect was obtained in each experiment for both batches of sonicate supernatant. This photochemical action spectrum differs therefore from those of other mixed-function oxidases which have been published (e.g. fig. 7) which show only one maximum, namely at 450 mμ. However, the remarkable constancy of the results for different sets of incubations performed on different days and using two separate batches of adrenal glands led to confidence in the result. Obviously it was desirable to obtain more points at other wavelengths than the five used here. Hence the investigation was extended to other wavelengths.

A feature of the calculation is that errors tend to be magnified at two stages. The first is in the calculation of K, where unity minus a number is divided by that number. The second is in the calculation of ΔK, where one number is subtracted from another of the same magnitude.

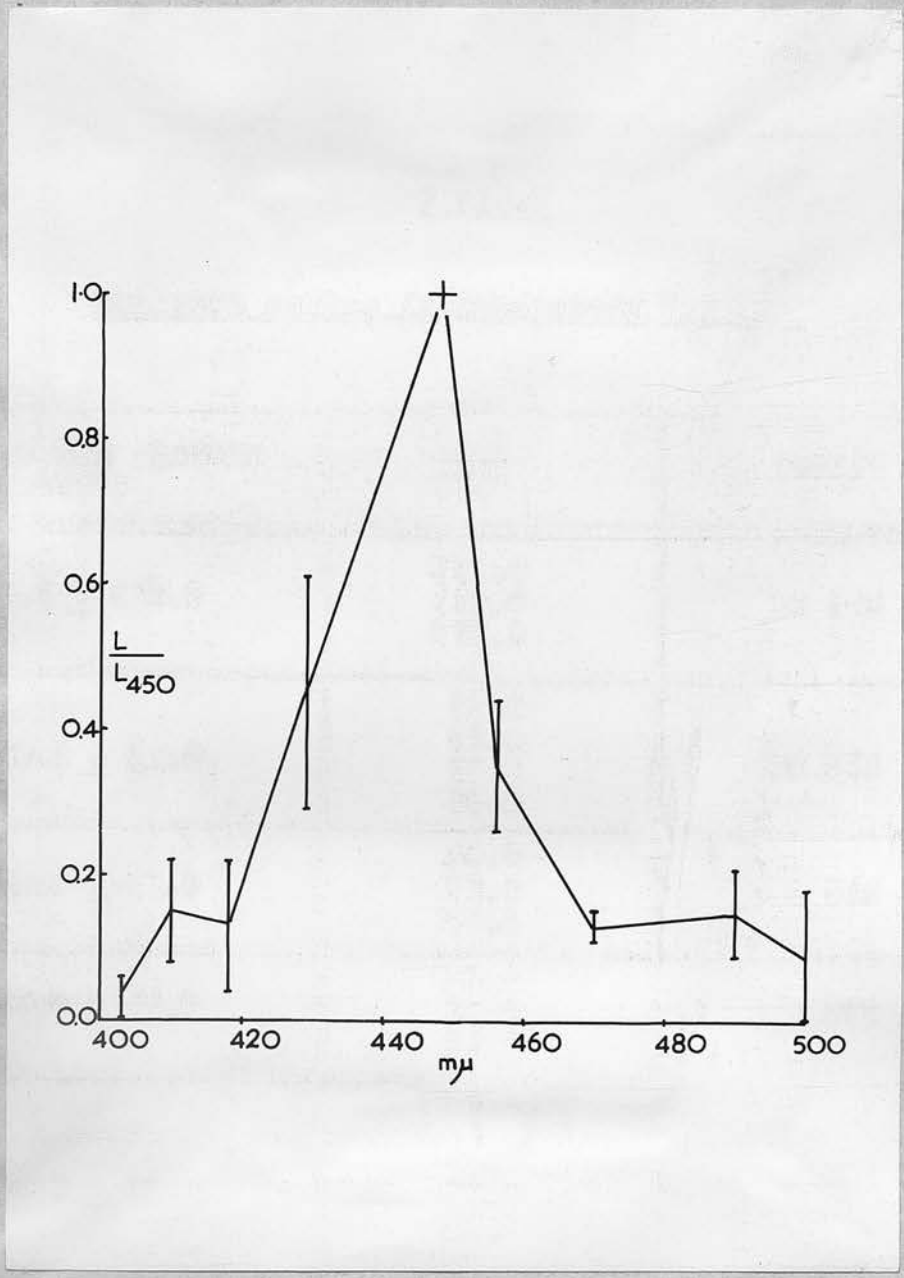


Fig.38. Photochemical action spectrum of light reversal of carbon monoxide inhibition of cholesterol side-chain cleavage (further experiments)

Table 9

Data for 2nd photochemical action spectrum

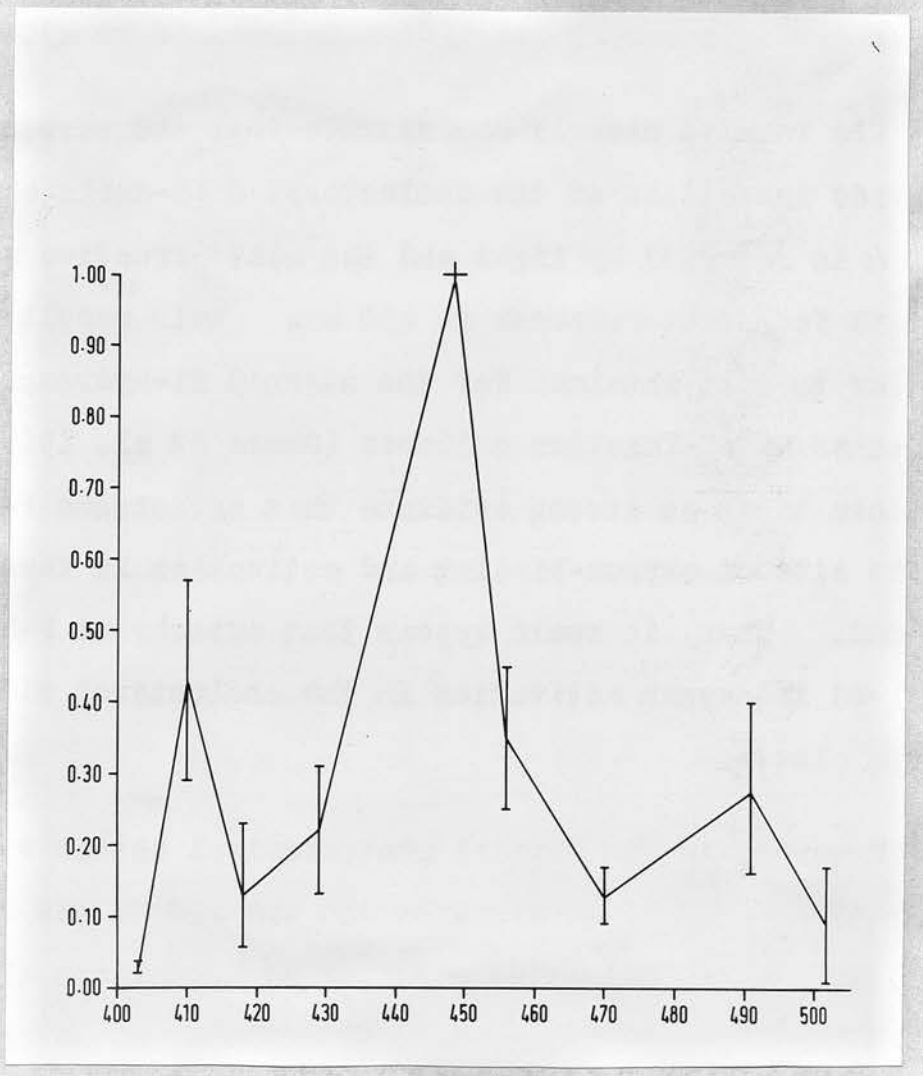
filter	$\frac{L}{L_{450}}$	average and standard error
403 mμ	0.033 0.023 0.027	0.028 ± 0.003
410 mμ	0.33 0.20 0.02 0.06	0.15 ± 0.07
418 mμ	-0.04 0.27 0.17	0.13 ± 0.09
429 mμ	0.64 0.58 0.13	0.45 ± 0.16
448 mμ	1.00	
456 mμ	0.21 0.32 0.51	0.35 ± 0.09
470 mμ	0.14 0.09 0.17	0.13 ± 0.02
491 mμ	0.13 0.32 0.11 0.04	0.15 ± 0.06
502 mμ	-0.09 0.15 0.20	0.09 ± 0.09

(4) The photochemical action spectrum of the cholesterol side-chain cleavage system - Further Experiments

For a more detailed investigation of the photochemical action spectrum, all nine interference filters were used. The experimental procedure was exactly as in section 3, that is one set of values for each wavelength was deter-:mined in each experiment. Three, and for some wavelengths, four, sets of values were obtained for each wavelength. Two separate batches of sonicate supernatant were used. This time the data was evaluated in a different way, in that $\frac{L}{L_{450}}$ was calculated separately for each determination, using the value for 450 mμ obtained for the particular experiment. Hence, the values for 450 mμ do not show a range, as they were made unity in each case.

Table 9 shows the data obtained, and fig. 38, the resulting photochemical action spectrum. It can be seen that this spectrum compared much more favourably with published action spectra of other mixed-function oxidases than the one obtained previously (fig. 37), in that there was one prominent peak with a maximum at 450 mμ, and only very minor secondary maxima at 410 mμ and 490 mμ.

Fig. 39 shows the photochemical action spectrum obtained with the combined data of Tables 8 and 9. It can be seen that the secondary maxima at 410 mμ and 490 mμ were still evident, but much less so than in fig. 37. However, by far the most effective wavelength for reversal



$\frac{L}{L_{450}}$

Fig. 39. Photochemical action spectrum of light reversal of carbon monoxide inhibition of cholesterol side-chain cleavage. (combined data of fig. 37 and fig. 38)

of the carbon monoxide inhibition is seen to be 450 mμ.

The results clearly demonstrate that the carbon monoxide inhibition of the cholesterol side-chain cleavage system is reversed by light and the most effective wavelength for light-reversal is 450 mμ. This result is similar to that obtained for the steroid 21-hydroxylase and other mixed-function oxidases (Omura et al, 1965), and has been taken as strong evidence that cytochrome P-450 is the site of oxygen-binding and activation in these systems. Thus, it would appear that cytochrome P-450 is involved in oxygen activation in the cholesterol side-chain system also.

However, in the initial photochemical action spectrum (fig. 37), light of wavelengths 410 and 490 mμ was found to be capable of effecting reversal of inhibition also, yet in the later spectrum (fig. 38), there was little evidence of this. The reason for this is not known. The only obvious difference between the preparations used in the two sets of experiments is that the K_d values used to obtain the spectrum of fig. 37 were lower (0.2-0.35) than those used to obtain the spectrum of fig. 38 (0.3-0.85).

(5) Summary

(a) The carbon monoxide inhibition of the cholesterol side-chain cleavage system is reversed by light.

(b) The most effective wavelength for light reversal is 450 mμ.

(c) In some preparations, light at 410 and 490 mμ was also effective in reversing the carbon monoxide inhibition, although not to the same extent as light at 450 mμ. The reason for this is not known, but while some preparations showed the effect clearly, others did not show it at all.

(d) The results strongly reinforce the hypothesis that cytochrome P-450 plays a role in the cholesterol side-chain cleavage system similar to that in the other mixed-function oxidases, namely as the site of oxygen binding and/or activation.

7. STUDIES ON THE RESOLUTION OF THE COMPONENTS OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE SYSTEM

(1) Introduction

The work so far described on the cholesterol side-chain cleavage system showed this system to be inhibited by carbon monoxide. This inhibition was reversed by light of wavelength 450 mμ, which suggested that the pigment called "P-450" was involved as the site of oxygen binding in the system. It was decided that in order to explore the system further, attempts should be made to purify the enzyme(s) involved.

Studies on the steroid 11β-hydroxylase of adrenal cortex mitochondria (Omura et al, 1965; Omura et al, 1966; Kimura and Suzuki, 1967) have shown that this system consists of at least three protein fractions - a flavoprotein, a non-haem iron protein, and a fraction containing P-450. On the basis of the similarity in behaviour of the two systems with respect to carbon monoxide inhibition and its reversal by light, it was considered quite possible that the cholesterol side-chain cleavage system could be resolved in a similar fashion.

Suggestive evidence that the cholesterol side-chain cleavage system might consist of several components was provided by a study of the relationship between the initial velocity of reaction versus concentration of supernatant of mitochondrial sonicate. Fig. 40 shows that the graph is non-linear, and that the initial

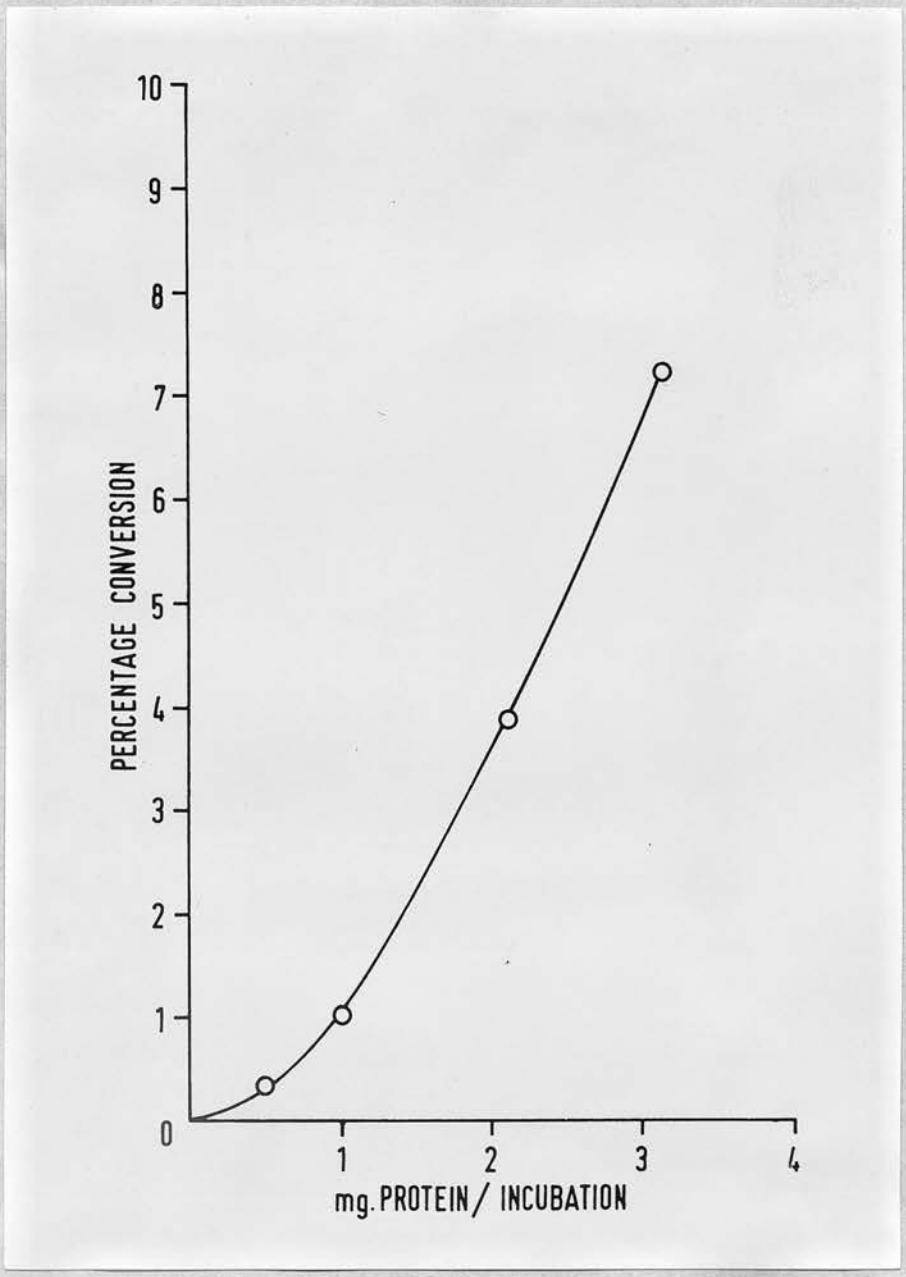


Fig.40(a) Plot of initial velocity against concentration of supernatant of mitochondrial sonicate.

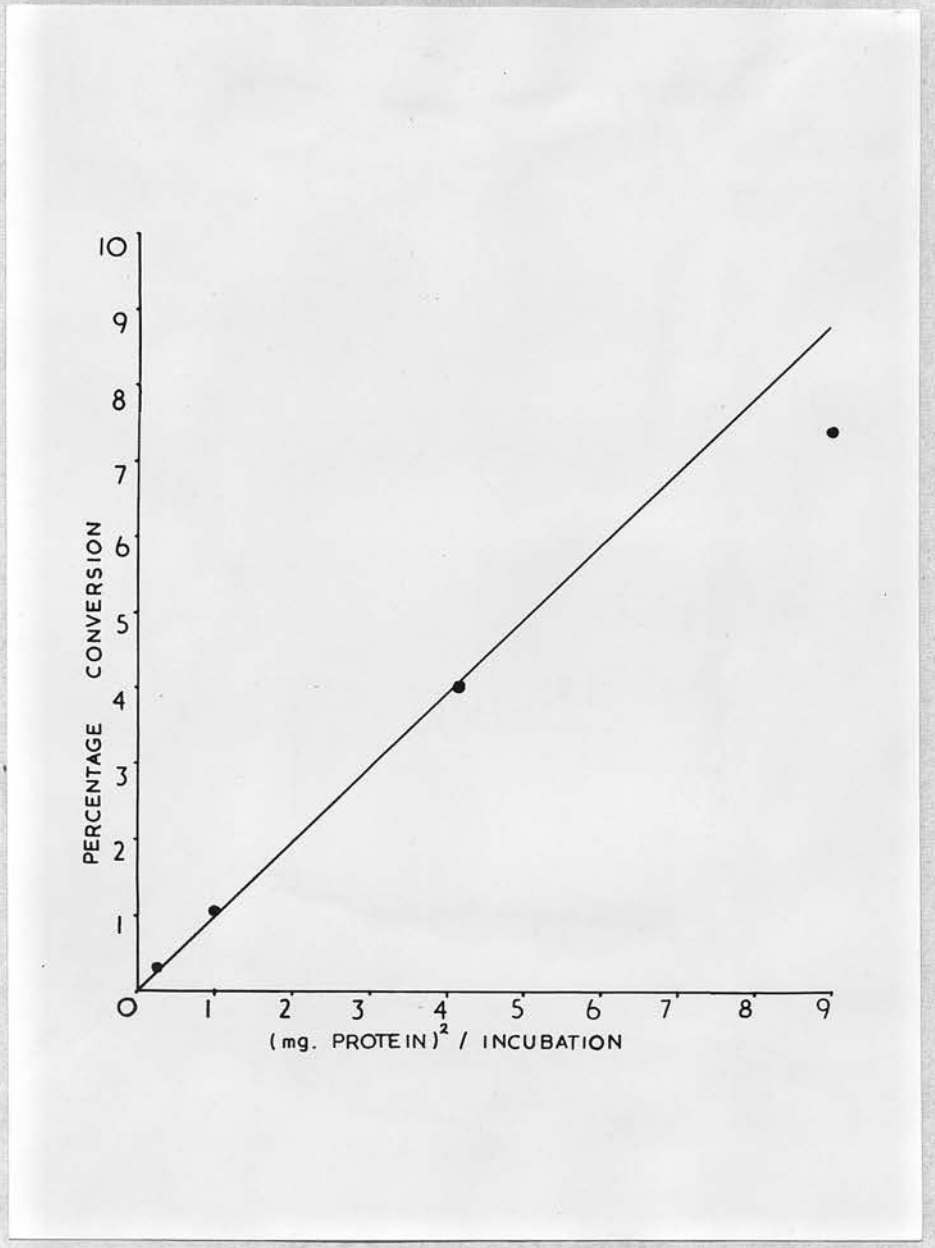


Fig.40(b). Plot of initial velocity against square of concentration of supernatant of mitochondrial sonicate.

velocity is closely proportional to the square of the enzyme concentration. This is suggestive of an enzyme system involving at least two dissociable proteins. Furthermore, studies by other laboratories had demonstrated that the system could be resolved into two protein fractions by ammonium sulphate fractionation of acetone powder extracts (Constantopoulos and Tchen, 1961a; Bryson and Kaiser, 1962). The work on the fractionation was begun therefore with this possibility in mind.

addition of ammonia solution. Again, the fractionation resulted in complete loss of activity.

The experiment was repeated a third time; this time solid ammonium sulphate was added, and the pH of the solution was continuously adjusted to 7.4 by addition of a few drops of ammonia solution. Incubations (15 min. duration, 37°C) were set up as below.

	Incubation	Percentage conversion
1	whole extract dialysed	9.7%
2	fraction A	< 0.5%
3	fraction B	< 0.5%
4	fractions A and B	0.7%

Thus, there appeared to be a trace of activity in the incubation containing the combined fractions but most of the activity was nevertheless lost in the fractionation procedure. In the paper of Constantopoulos and Tchen (1961a), the activity in the recombined fractions was quite low, but then the activity in the controls was also lower than in our preparations.

Because of the lack of success with fractionation attempts using ammonium sulphate, it was decided to explore other methods of fractionation.

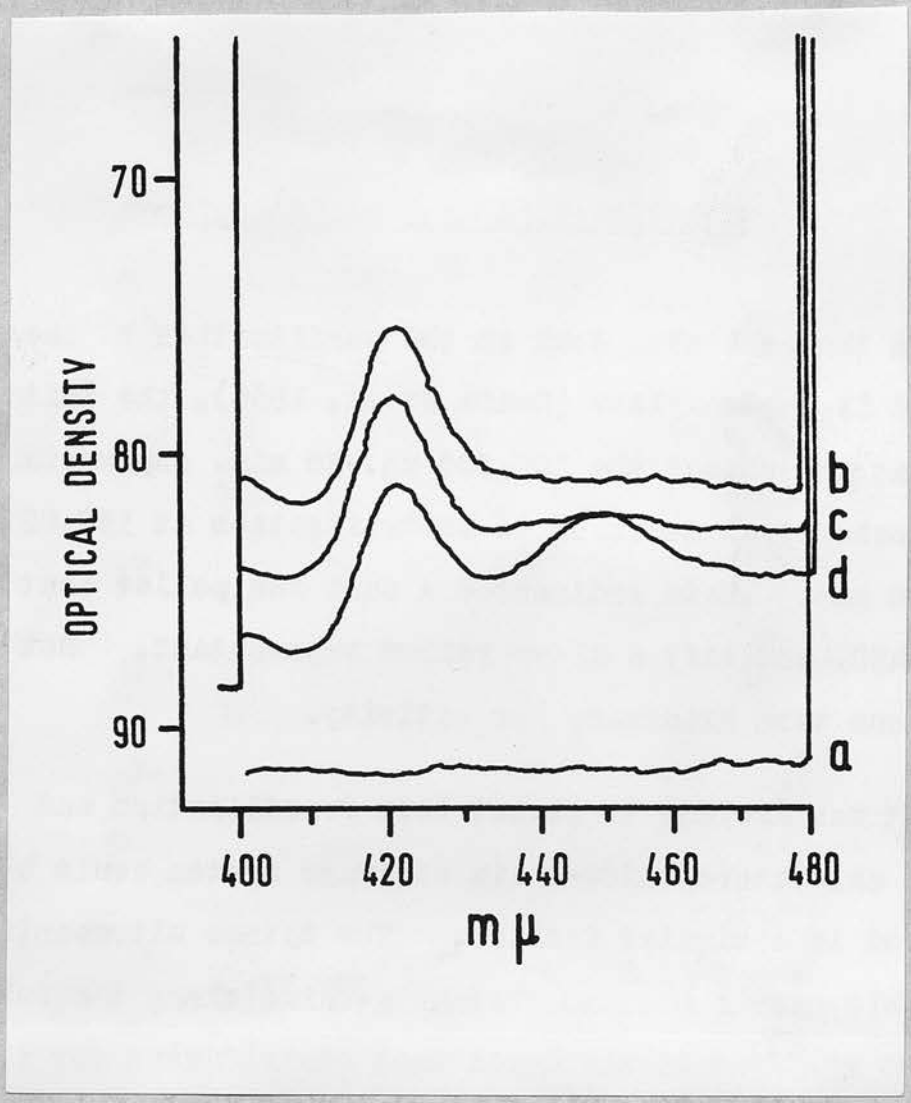


Fig.41. NADPH-reduced carbon monoxide difference spectrum of centrifuged fractions of supernatant of mitochondrial sonicate.

(a) 190min.-105,000g supernatant, plus NADPH-generator, in both cells.

(b) as (a), carbon monoxide bubbled through experimental cell for 30sec.

(c) as (b), sample of 190min.-105,000g pellet added to both cells.

(d) as (c), sample of 60min.-105,000g pellet added to both cells.

(3) Fractionation by Centrifugation

In the published work on the purification of the steroid 11β-hydroxylase (Omura et al, 1966), the initial step was to subject the 105,000 xg.-30 min. supernatant of mitochondrial sonicate to centrifugation at 150,000 xg. for 100 min. This sedimented a dark red pellet containing the P-450, and left a clear yellow supernatant. Both fractions were necessary for activity.

It was decided to repeat this fractionation and see if the cholesterol side-chain cleavage system could be resolved in a similar fashion. The Spinco ultracentrifuge available gave a maximum average g-value along the tube of 105,000 xg., but it was hoped that centrifuging for 150 min. would have a similar effect as centrifuging at 150,000 xg. for 100 min., as the product of g-value versus time would be the same.

The 105,000 xg.-30 min. supernatant of mitochondrial sonicate prepared as described in Chapter 3, was a yellow-brown, cloudy, opalescent liquid. When centrifuged for 60 min. at 105,000 xg., a ruby-red pellet was obtained. When the supernatant from this was centrifuged for a further 190 min. at 105,000 xg., a loosely packed, almost colourless pellet and a clear yellow supernatant were obtained. Fig. 41 shows the reduced carbon monoxide difference spectrum of the 190 min. supernatant, the 190 min. supernatant plus the 190 min. pellet, and

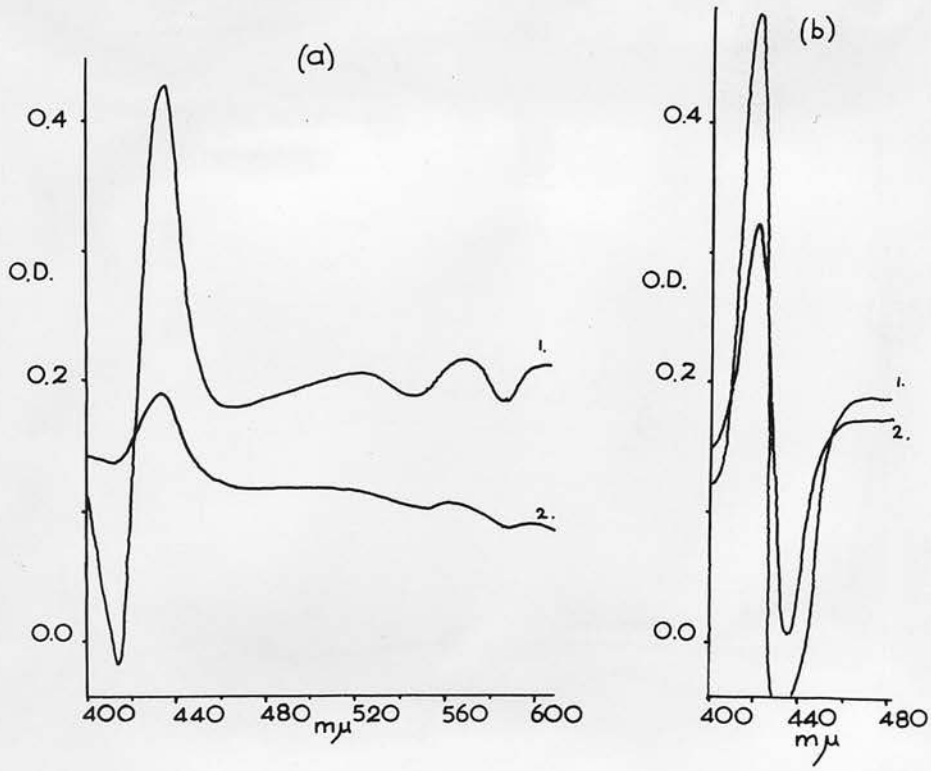


Fig.42. (a) Aerobic minus anaerobic difference spectra

1. human blood in distilled water.
2. 190min.-105,000g supernatant of mitochondrial sonicate.

(b) Difference spectra, dithionite-reduced plus carbon monoxide, minus dithionite reduced.

1. human blood in distilled water.
2. 190min.-105,000g supernatant of mitochondrial sonicate.

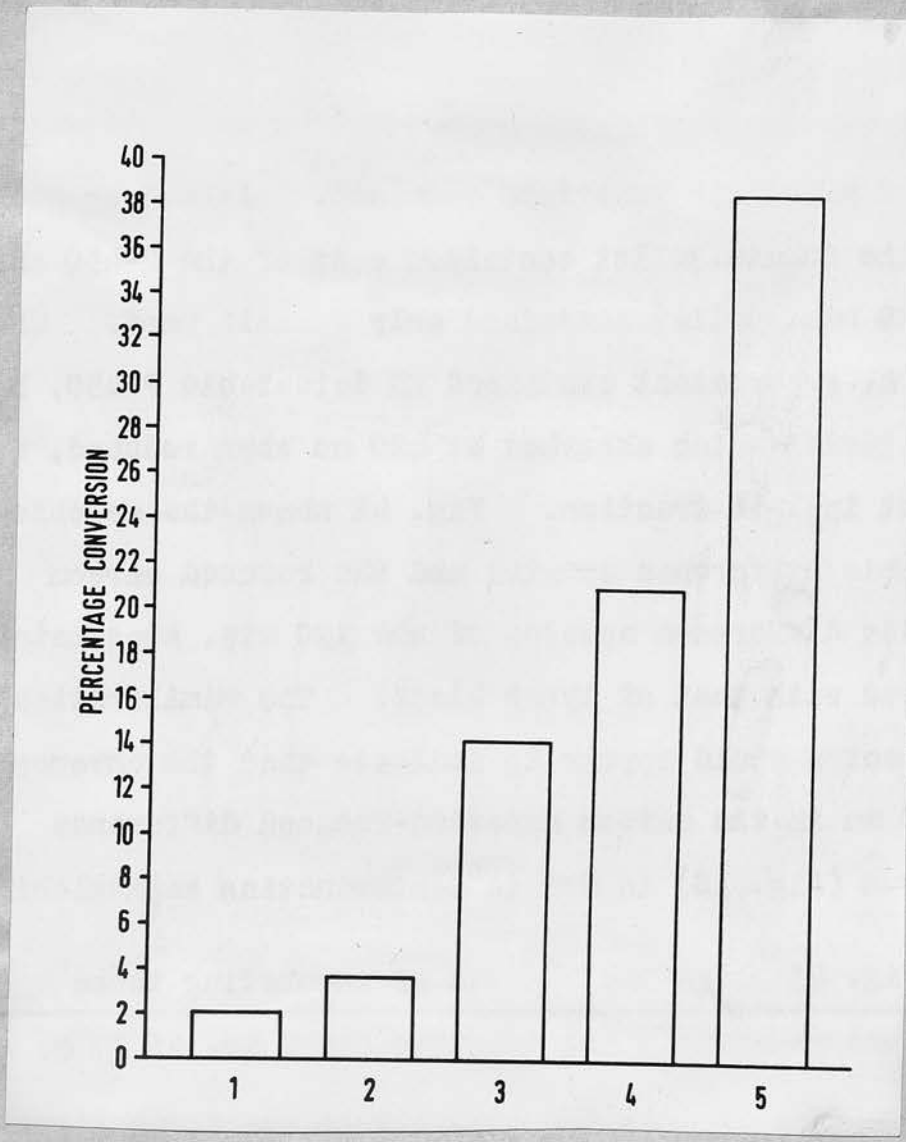


Fig.43. Cholesterol side-chain cleavage activity of centrifuged fractions of supernatant of mitochondrial sonicate.

1. 60min.-105,000g pellet.
2. 190min.-105,000g pellet.
3. 190min.-105,000g supernatant.
4. 190min.-105,000g supernatant plus 190min.-105,000g pellet.
5. all three fractions.

that of all three fractions combined. It can be seen that the 60 min. pellet contained most of the P-450 and the 190 min. pellet contained only a small part. The 190 min. supernatant contained no detectable P-450, but the material which absorbed at 420 mμ when reduced, was present in this fraction. Fig. 42 shows the aerobic-anaerobic difference spectra and the reduced carbon monoxide difference spectra of the 190 min. supernatant compared with that of lysed blood. The similarities in the spectra would appear to indicate that the chromophore at 420 mμ in the carbon monoxide-reduced difference spectrum (fig. 18) is due to contaminating haemoglobin.

Fig. 43 shows the results of incubating these fractions separately and together for 2 hr. at 37°C. The results show that the activity in the incubation with all three fractions combined is double that of the sum of the activities of each incubated separately. This would imply that a partial resolution into at least two fractions had been achieved. As the 60 min. and the 190 min. pellets contained all the detectable P-450 and the supernatant presumably contained an NADPH-cytochrome P-450 reductase by analogy with the work of Omura et al (1966), it could be that the cholesterol side-chain cleavage system consists of those components also. However, fig. 43 shows that the 190 min. supernatant contained more than one-third of the activity of the recombined preparation although it contained no

detectable P-450. As other evidence to be discussed implicated P-450 in the side-chain cleavage reaction, it could be that only a fraction of the 'total P-450' participates in the side-chain cleavage reaction. This fraction was below the limit of resolution of the spectrophotometer and was distributed throughout the three fractions obtained by centrifugation, which could presumably be artefacts of the sonication process.

(4) Fractionation of the sonicate 105,000 xg.-30 min. supernatant with Sephadex G-100

As it did not seem possible to fractionate the system further by using centrifugation, it was decided to try column chromatography. As molecular sieve filtration using Sephadex is a very mild technique it was decided to attempt fractionation with this product. Also, as one of the components of the cholesterol side-chain cleavage system is, at least in part, sedimented by prolonged ultracentrifugation and consequently has a very high particle weight, it was hoped that chromatography based on separation by particle weight would achieve resolution of some of the components.

20 g. Sephadex G-100 was left in 0.02M KCl for 14 days with a change of salt solution every 2-3 days to allow complete swelling and removal of fines. Glass columns were made with an internal diameter of 2.5 cm. The bottom of the column was plugged with a Teflon disc fitted with a Neoprene O-ring. A short length of 2 mm. internal diameter Teflon tubing was inserted into a hole drilled in the middle. On top of the disc was placed a small wad of glass wool and a layer of coarse washed sand 0.5 cm. thick. The column was filled by the method of Gelotte (1964), and allowed to run overnight at a flow rate of some 7.5 cm./10 min. to pack the Sephadex gel. The final height of the gel column was 25 cm. A 2.5 cm. diameter disc of very fine mesh stainless steel was

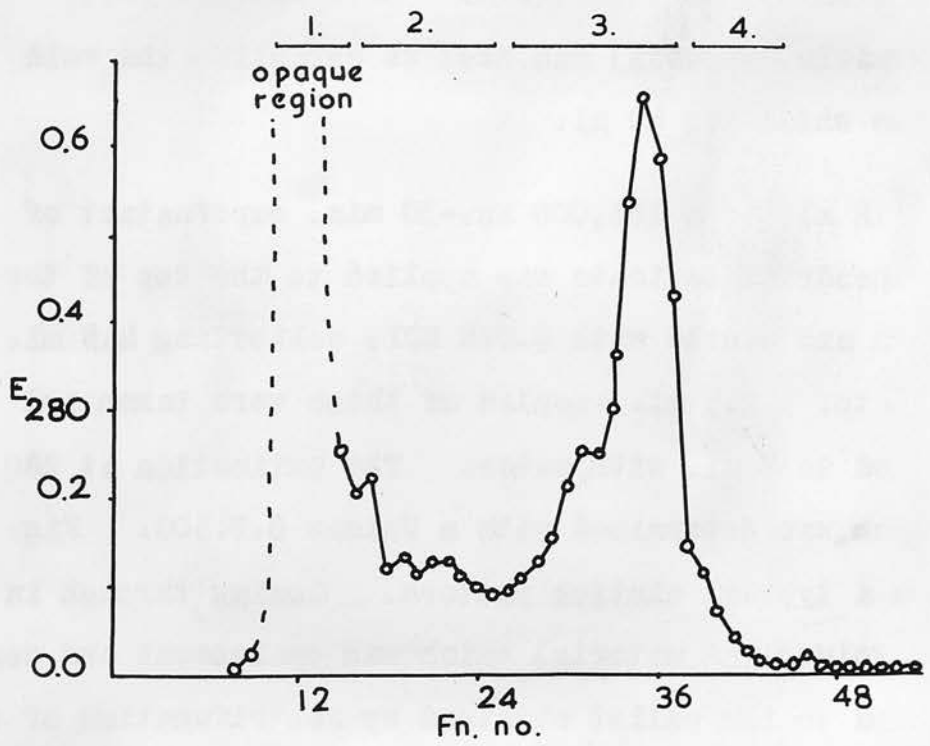


Fig.44. Elution pattern of chromatograghy of 30min.-105,000g supernatant of mitochondrial sonicate on Sephadex G 100. Volume of fractions, 4.5ml. Fractions were pooled into four combined fractions as shown.

placed on top of the column. Blue Dextran 2000 (Pharmacia, Uppsala) was used to establish the void volume which was 40 ml.

12 ml. of a 105,000 xg.-30 min. supernatant of mitochondrial sonicate was applied to the top of the column and eluted with 0.02M KCl, collecting 4.5 ml. aliquots. 0.5 ml. samples of these were taken and diluted to 3 ml. with water. The extinction at 280 mμ in each was determined with a Unicam S.P.500. Fig. 44 shows a typical elution pattern. Coming through in the void volume was material which was opalescent and corresponded to the pellet obtained by centrifugation of the material at 105,000 xg. for 60 min. Fractions were pooled into four combined fractions as shown in fig. 44. These were concentrated to 6 ml. each by dialysing against a thick paste of polyethylene glycol ('Carbowax', Union Carbide) in 0.02M KCl, and 0.5 ml. samples were incubated for 2 hr. under standard conditions. The results were:-

Incubation	Percentage conversion
combined fraction 1	2.3
combined fractions 1 + 2	5.8
combined fractions 1+2+3+4	5.0
combined fractions 2+3+4	0.8

This shows that the activity resided in combined fractions 1 plus 2. Addition of combined fractions 3 plus 4 apparently reduced the activity slightly. Without combined fraction 1, there was very little activity, but

this component had some activity by itself. Thus, the columning procedure has achieved partial resolution of the system into two components. However, the activity of the fractionated material was very low compared to that of unfractionated preparations.

(5) Fractionation of acetone powder extracts with Sephadex G-200

Because the sonicate 105,000 xg.-30 min. supernatant contained a component which was particulate, the experiments described in the last section were repeated for extracts of acetone powder, which appear optically clear. This time, however, Sephadex G-200 was used and was allowed to swell for 14 days in 0.1M phosphate buffer before use. Extinction at 280 mμ was monitored by means of an L.K.B. 8300A "Uvicord II" absorptiometer and 6520H recorder. The fraction collector was an L.K.B. 7000A "UltroRac".

150 mg. acetone powder was extracted with 10 ml. 0.1M phosphate buffer and applied to a column of the same dimensions as previously used for the Sephadex G-100 chromatography. Elution was with 0.1M phosphate buffer. Fig. 45a (top diagram) shows a typical elution pattern. This was similar to that obtained in the case of super-:natant of mitochondrial sonicate (fig. 44) except that the first peak was much smaller owing to the fact that there was apparently no particulate material in the extract. Combined fractions 1 and 2 were taken as shown and concen-:trated with 'Carbowax' to 5 ml. 1 ml. was taken for incubation(60 min., 37°C); that is, corresponding to twice the material used for a standard incubation. 1 ml. of the original extract was used as control.

The results were:-

Incubation	Percentage conversion
CONTROL	47.0
combined fraction 1	1.7
combined fraction 2	2.4
combined fractions 1+2	8.8

Thus again there was partial resolution into two components, and again the fractionation resulted in considerable loss of activity. Once more it was found that addition of the material in the last E_{280} peak to an incubation did not increase the activity, and, if anything, slightly decreased it. Later it was found that if the material was dialysed prior to application to the column, this last peak was no longer present indicating it consisted of low molecular weight material, possibly nucelotide phosphates.

It was decided to repeat the whole procedure on a bigger scale. A G-200 column 4.5 cm. in diameter and 25 cm. long was prepared in 0.04M phosphate buffer. A second column of DEAE Sephadex A-25 was also prepared. This was 2.5 cm. diameter and 25 cm. long. The ion exchanger was left overnight in 0.04M phosphate buffer before packing the column.

The following parameters were measured in the eluate fractions in order to furnish information as to the

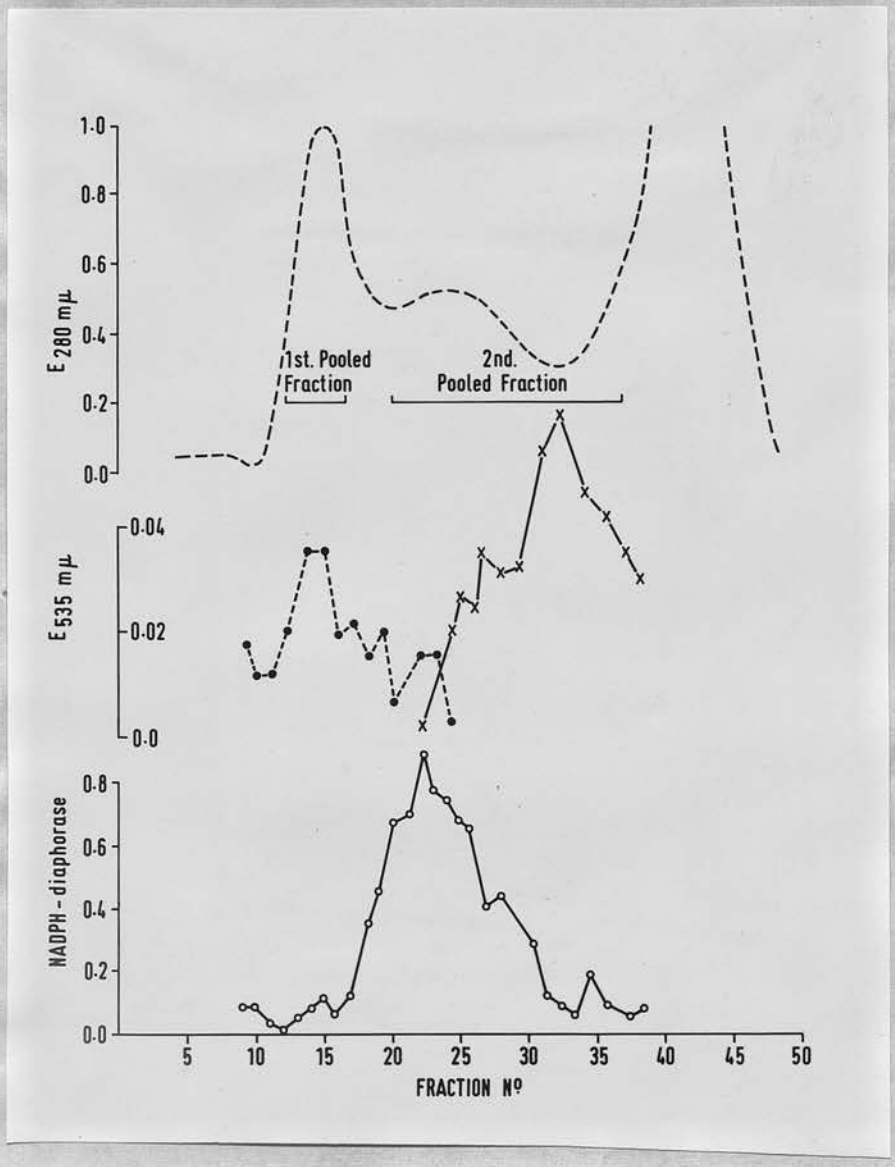


Fig. 45.

------ E_{280}

●····● haem iron

×——× non-haem iron

○——○ NADPH-diaphorase

(a) Elution pattern of acetone powder extract chromatographed on Sephadex G 200, showing distribution of haem iron, non-haem iron and NADPH-diaphorase.

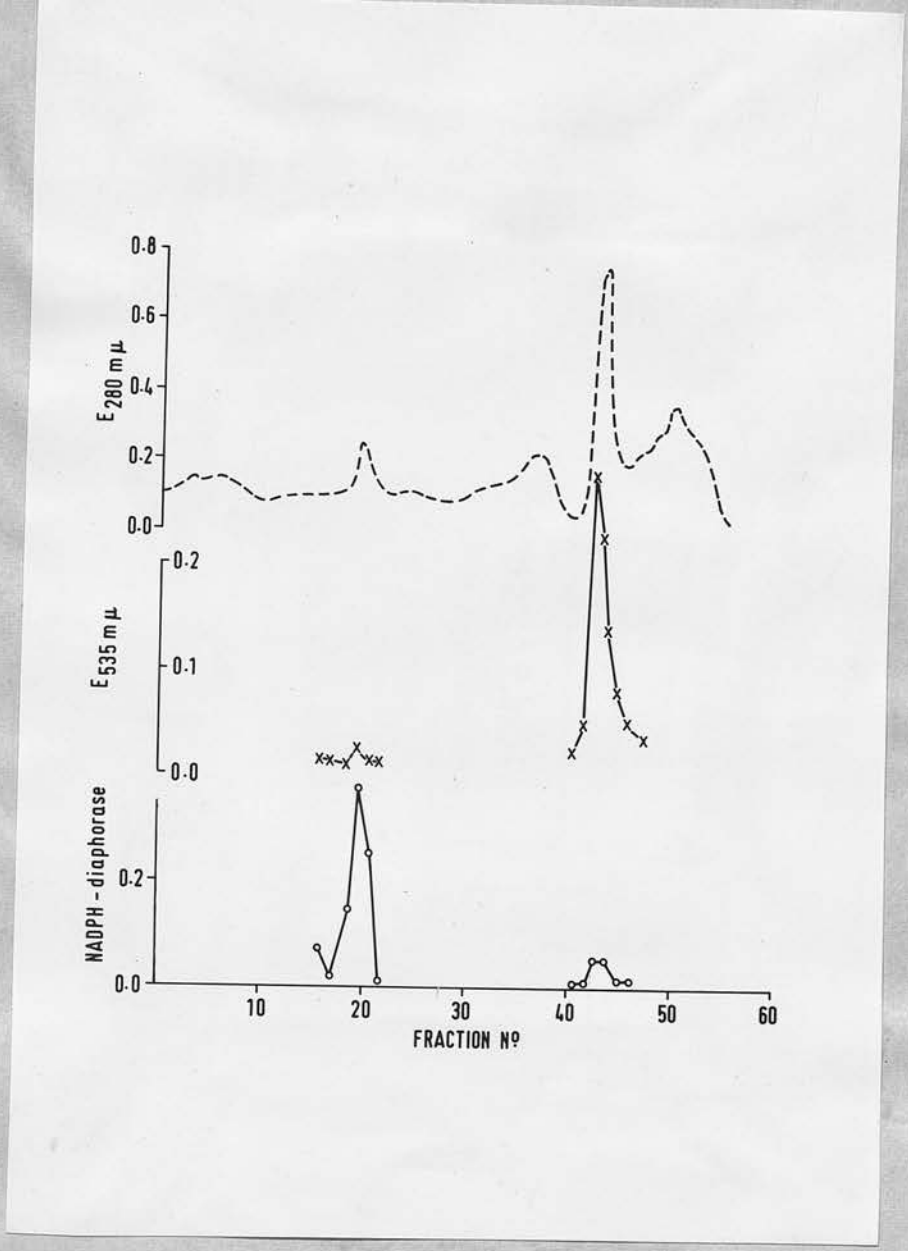


Fig. 45 (contd.)

(b) Elution pattern of 2nd. pooled fraction from (a) on DEAE-Sephadex A 25, showing distribution of NADPH-diaphorase and non-haem iron.

nature of the proteins being fractionated - haem iron, non-haem iron and NADPH-diaphorase activity (see Appendix 3).

600 mg. acetone powder was extracted with 45 ml. 0.1M phosphate buffer and applied to the G-200 column. Elution was with 0.04M phosphate buffer. The flow rate was 15-20 ml./10 min. and 10 ml. cuts were taken. Fig. 45a shows the elution pattern of the three parameters. As can be seen, the haem iron corresponded to the first E_{280} peak, followed by fractions containing NADPH-diaphorase and finally by fractions containing non-haem iron. The figure also shows how the fractions were pooled. The second combined fraction was run on to the DEAE-Sephadex column. As this was taking place, a brown band gradually accumulated on the top of the column. A salt gradient was set up as follows:- Into one 500 ml. conical flask was placed 250 ml. 0.04M phosphate buffer. Into a second was placed 250 ml. 0.04M phosphate buffer containing 0.7M sodium chloride. These flasks were connected by a siphon. The first flask led into the DEAE-Sephadex column and contained a magnetic stirrer. The flow rate was 40 ml./hr. Fig. 45b shows the elution pattern and the regions containing the NADPH-diaphorase activity and non-haem iron. The fractions containing the non-haem iron were reddish-brown in colour and corresponded to the brown band on top of the column at the start of the run. This material appeared from the foot of the column shortly before the

eluting buffer was exhausted. This elution pattern is very similar to that obtained by Omura et al (1966) for the chromatography of sonicate 150,000 xg.-100 min. supernatant on DEAE cellulose. The first combined fraction was found subsequently to contain the P-450 present in the acetone powder extract (fig. 51a). Thus this corresponds to the sonicate 150,000 xg.-100 min. pellet of these authors. The combined fractions contain-:ing NADPH-diaphorase activity and those containing non-haem iron were concentrated against 'Carbowax', dialysed against 0.04M phosphate buffer, and incubated in the following manner:-

Incubation No.				
1	2	3	4	5
P-450 fraction	P-450 fraction + NADPH-diaphorase	P-450 fraction + non-haem iron protein	NADPH-diapho-:rase + non-haem iron pro-:tein	all three fract-:ions

Each incubation contained material corresponding to 60 mg. acetone powder, i.e. four standard assay incubations. The final volume was 5 ml. and the incubation time was 1 hr. The results were:-

Incubation	Percentage conversion
1	27.6
2	35.1
3	72.5
4	6.1
5	72.3

This demonstrated that the maximum activity lay in a combination of the P-450 fraction and the non-haem iron protein and in all three fractions together. There was also considerable activity in the P-450 fraction by itself, more in a combination of the P-450 fraction and the NADPH-diaphorase, and a little in the diaphorase and non-haem iron fractions together. However, the conversion rates were so great that by the time the incubations were stopped, the rate of side-chain cleavage would have slowed down to well below the initial velocity. Some of the above incubations were therefore repeated, but the incub-:ation time was cut to 30 min.; also the concentration of the P-450 fraction was halved, but the concentration of the other fractions was kept the same.

The results were:-

Incubation	Percentage conversion
1. P-450 fraction	1.4
2. P-450 fraction + non-haem iron protein	21.1
3. All three fractions	28.2

Under these conditions the P-450 fraction possessed practically no activity of itself, and all three fractions were required for maximum activity. However, a combination of the P-450 fraction and the non-haem iron protein also possessed considerable activity. The results suggest that all three protein components might have been required

for activity, but the NADPH-diaphorase was a major contaminant of the P-450 fraction. There was also a certain degree of contamination of all three components with respect to one another, but this was only manifest at high concentrations.

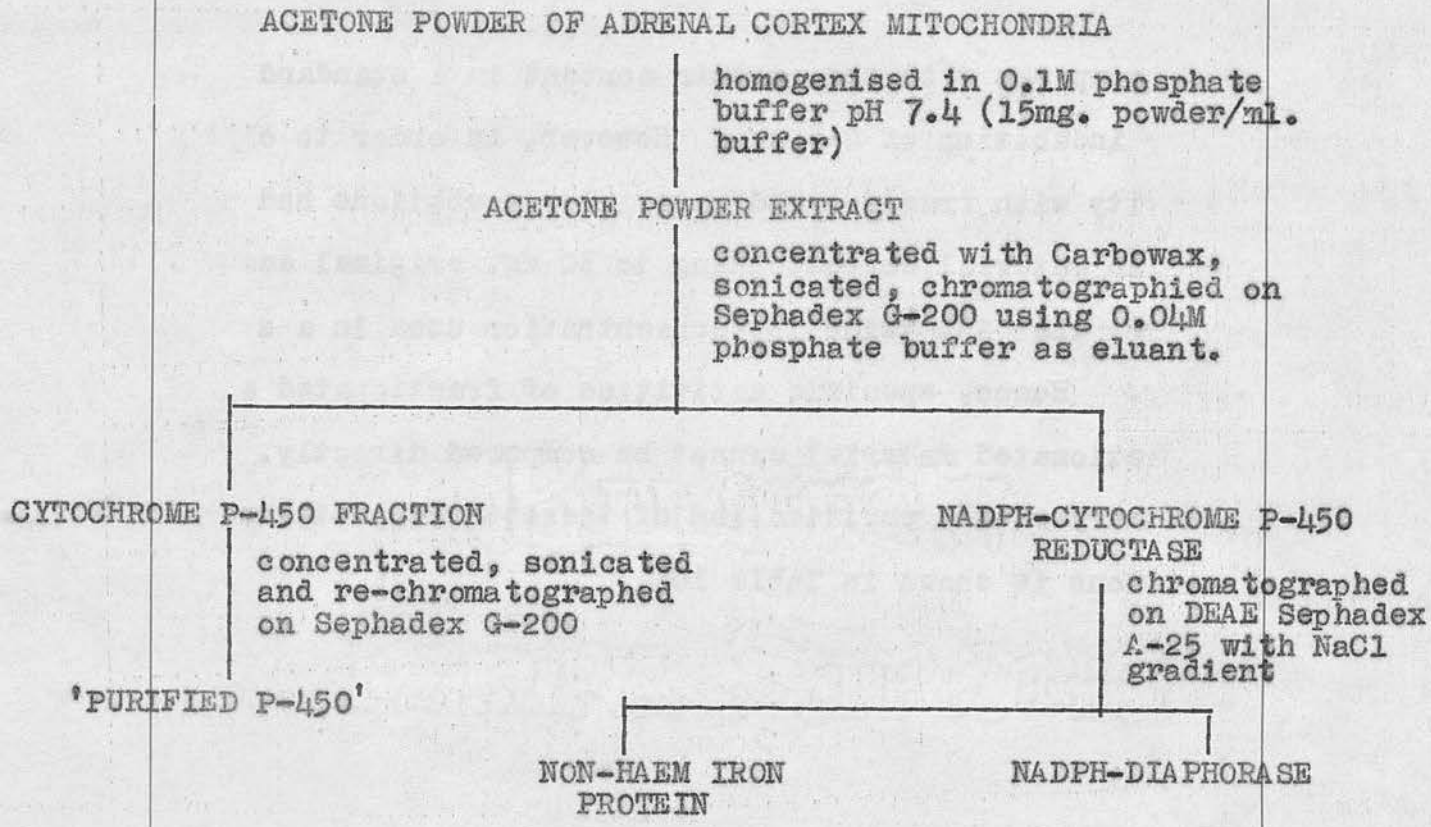
The experiment was therefore repeated as follows:- 1800 mg. acetone powder was extracted with 130 ml. 0.1M phosphate buffer and concentrated to 40 ml. with 'Carbowax'. This took 3 hr. The material was then sonicated for 5 min. at 0°C and applied to the G-200 column. The P-450 combined fraction was concentrated to 40 ml. with 'Carbowax'. The P-450 fraction was then sonicated once more for 5 min. and rechromatographed on the G-200 column. The centre fractions were pooled and concentrated once more to 40 ml. with 'Carbowax'. The NADPH-diaphorase and non-haem iron protein were dialysed against 0.04M phosphate buffer. Incubations (15 min., 37°C) were set up as follows:- Incubations 1-5 contained material corresponding to 30 mg. original acetone powder, i.e. two standard assay incubations, whereas incubations 6-8 contained material corresponding to one standard assay incubation. The results were:-

	Incubation	Percentage conversion
1.	P-450 fraction.	1.0%
2.	P-450 fraction + NADPH-diaphorase.	0.8%
3.	P-450 fraction + non-haem iron protein.	4.1%
4.	NADPH-diaphorase + non-haem iron protein.	3.4%
5.	P-450 fraction + NADPH-diaphorase + non-haem iron protein.	18.5%
6.	P-450 fraction + non-haem iron protein.	$<$1.0%
7.	NADPH-diaphorase + non-haem iron protein.	$<$1.0%
8.	P-450 fraction + NADPH-diaphorase + non-haem iron protein.	$<$1.0%

This time the results show clearly that all three components were required for activity, the activity in any two compo-:nents together being less than 25 per cent of the activity of all three components combined. However, this result was only observed if the components were incubated at concentrations which are twice those that would be present in the standard assay. If incubated at concentrations equal to those used in the standard assay, no activity was observed in any of the incubations. Perhaps this critical concentration dependence was responsible for the low recoveries of activity observed in earlier attempts at chromatography. The results also indicate that the sonication procedure resulted in decreased cross-contamination of components. The protein content for each component in incubation 5 was as follows:-

Table 10

Purification of components of cholesterol side-chain cleavage system



P-450 fraction 0.60 mg.
NADPH-diaphorase 0.35 mg.
Non-haem iron protein 0.17 mg.

Total protein content 1.1 mg.

This compares with the protein content in a standard assay incubation of 4-5 mg. However, in order to obtain activity with fractionated material, incubations had to contain material corresponding to 30 mg. original acetone powder, that is, twice the concentration used in a standard assay. Hence, specific activities of fractionated and unfractionated material cannot be compared directly.
A summary of the purification of these three protein fractions is shown in Table 10.

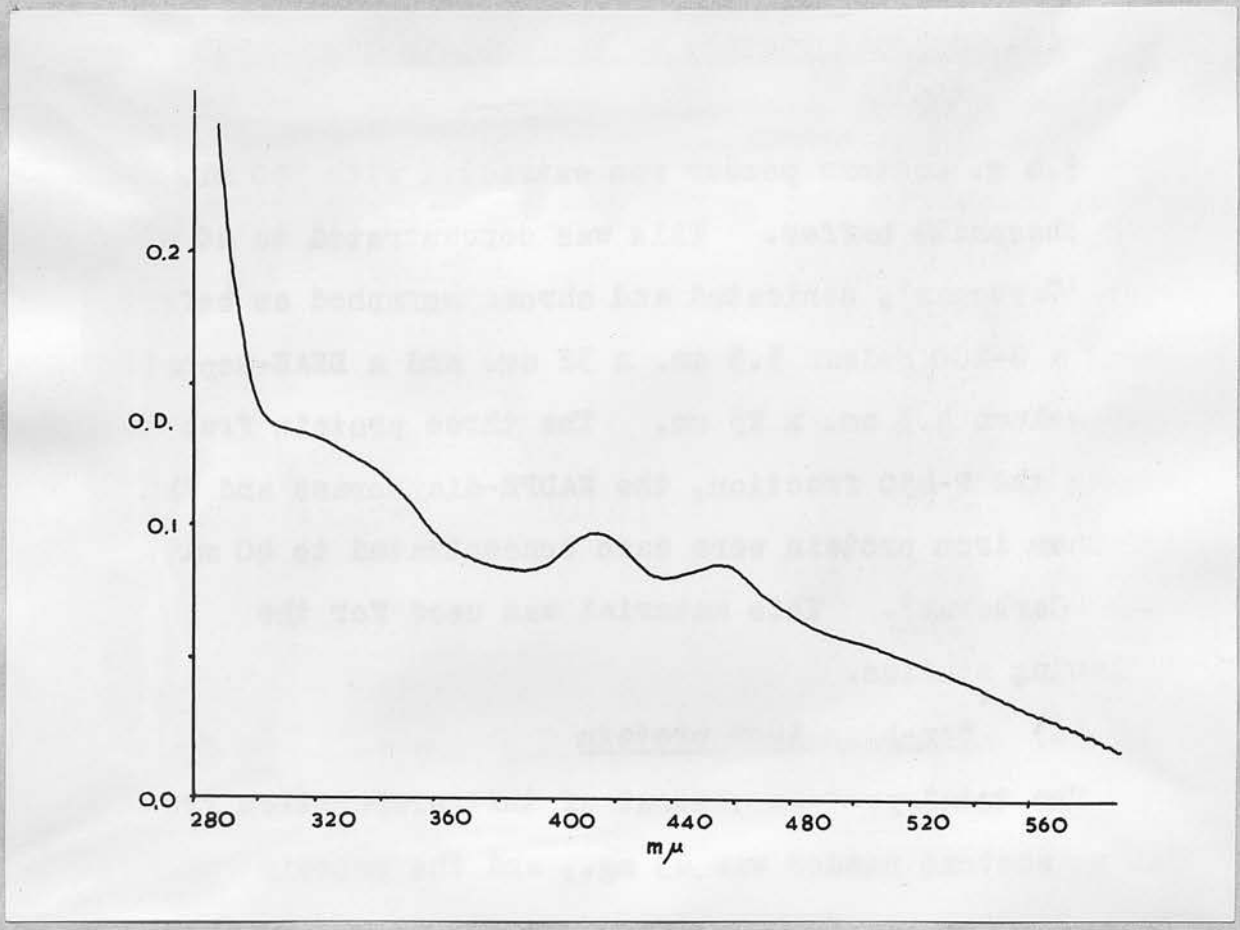


Fig.46. Absorption spectrum of non-haem iron protein involved as a component of the cholesterol side-chain cleavage system.

exptl. cell - sample of protein in 0.04M phosphate buffer.

reference cell - 0.04M phosphate buffer.

(6) Characteristics of the Protein Components

5.4 g. acetone powder was extracted with 390 ml. 0.1M phosphate buffer. This was concentrated to 40 ml. with 'Carbowax', sonicated and chromatographed as before, using a G-200 column 5.5 cm. x 32 cm. and a DEAE-Sephadex A-25 column 4.5 cm. x 25 cm. The three protein fractions, namely the P-450 fraction, the NADPH-diaphorase and the non-haem iron protein were each concentrated to 40 ml. with 'Carbowax'. This material was used for the following studies.

(i) Non-haem iron protein

The total protein content of this preparation from 5.4 g. acetone powder was 43 mg., and the protein concentration was 0.54 mg./ml. As discussed previously, the preparation was reddish-brown in colour and contained acid-extractable iron. When a sample was added to a little dilute hydrochloric acid, the odour of hydrogen sulphide was detected, indicating the presence of acid-labile sulphur in the protein. These properties are characteristic of the non-haem iron protein of adrenal cortex which has been called "adrenodoxin" (Suzuki and Kimura, 1965). Fig. 46 shows the absorption spectrum of the component. This was very similar to the absorption spectrum of oxidised adrenodoxin (Kimura and Suzuki, 1967), with maxima at 455 and 414 mμ, and a broad maximum at 320 mμ. The iron content of the material was measured as before, but quantitated, using ferrous ammonium

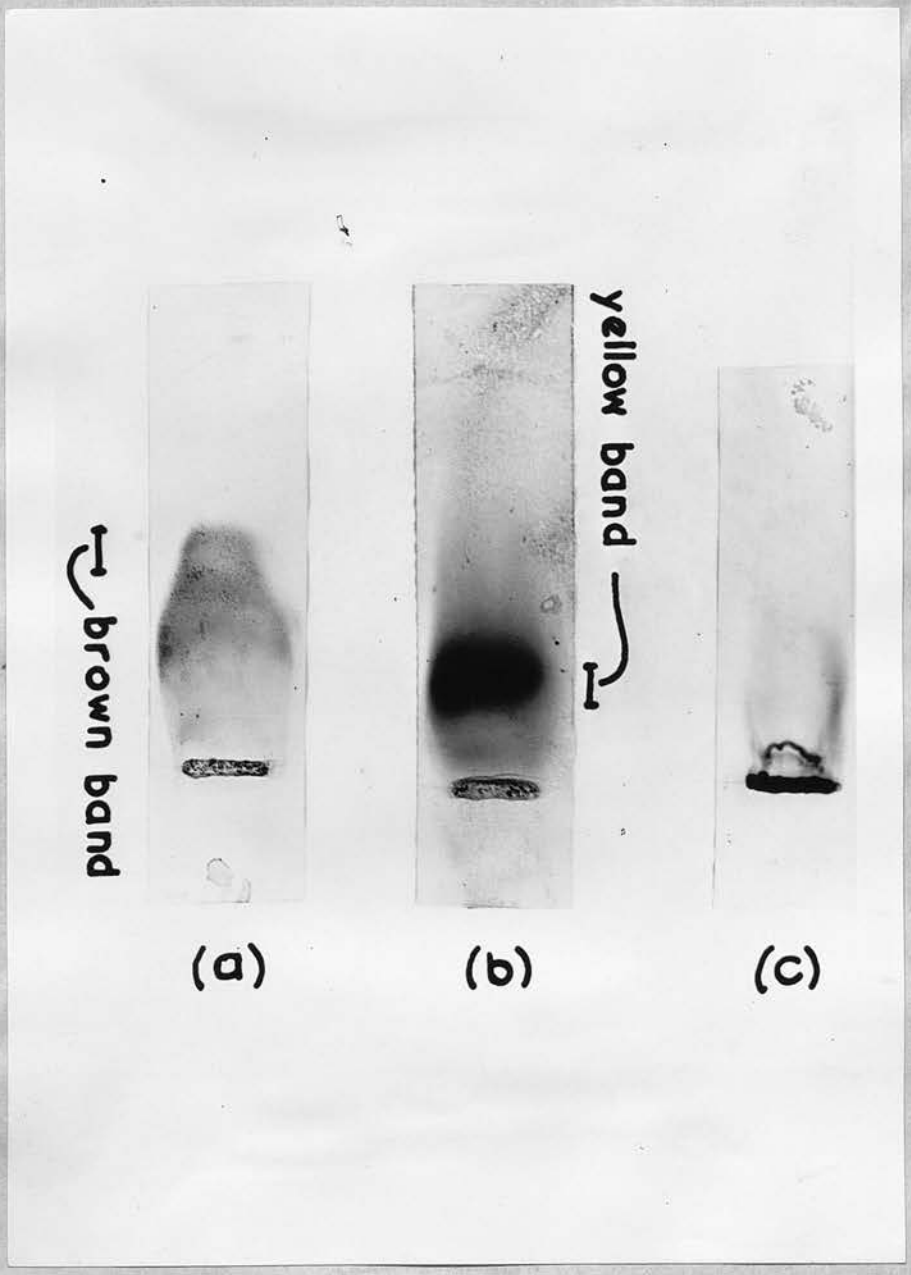


Fig.47. Cellulose acetate electrophoresis of isolated protein fractions. Barbitone buffer 0.07M pH8.4 Potential difference 200v. Stained with Amido black.

(a) non-haem iron protein. Duration of run 45min.

(b) NADPH-diaphorase. Duration of run 165min.

(c) P-450 fraction. Duration of run 165min.

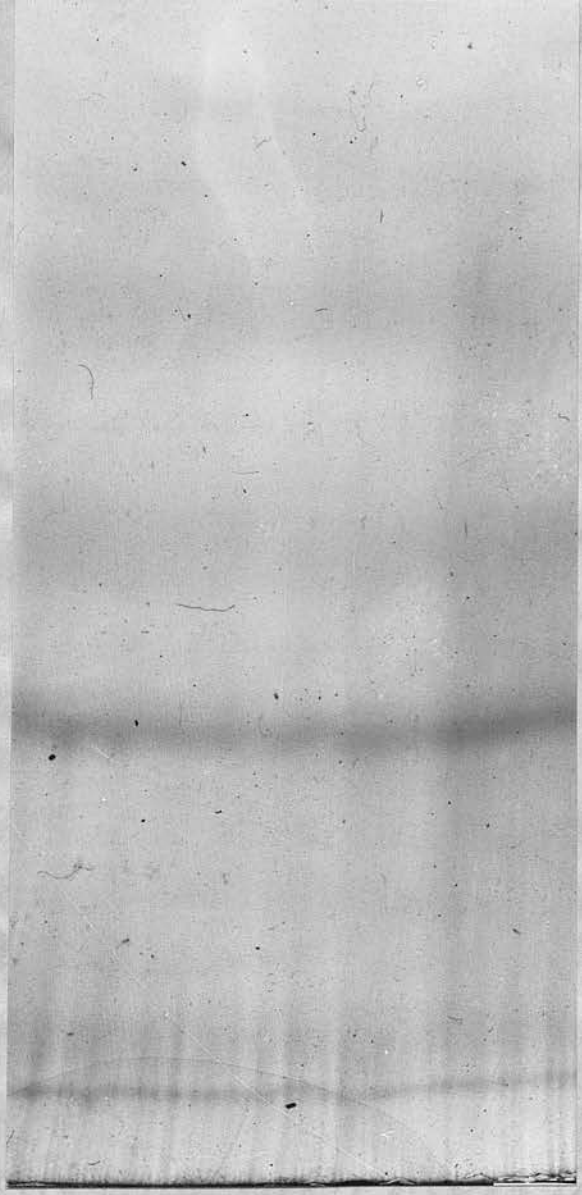


Fig .48. Polyacrylamide gel electrophoresis of non-haem iron protein.

Phosphate buffer 0.1M pH7.4. Potential difference 3v/cm.

Duration of run 6hr.

Stained with Amido black.

sulphate as standard.

The result was:-

1 mole iron/12,050 g. protein

Kimura and Suzuki (1967) consider adrenodoxin to contain two atoms of iron per molecule of weight 15,000-20,000. Thus, it appears our preparation is not completely pure.

Electrophoresis

Zone electrophoresis of the non-haem iron protein was conducted on a strip of cellulose acetate in barbitone buffer pH 8.6, 0.07M as described by Kohn (1960). After 45 min. the strip was dried and stained with Amido Black. Fig. 47a shows that the preparation contained at least three protein components, the fastest running of which was the characteristic brown colour of adrenodoxin.

Zone electrophoresis in polyacrylamide gel was also performed after the method of Cruft (1962), using phosphate buffer pH 7.4 and a potential difference of 3v/cm. The duration of the run was 6 hr. Fig. 48 shows that there was one major component and three minor ones.

The non-haem iron protein was found to be very unstable to freezing - this procedure resulted in bleaching of the protein. It could, however, be stored at 0°C for about 10 days.

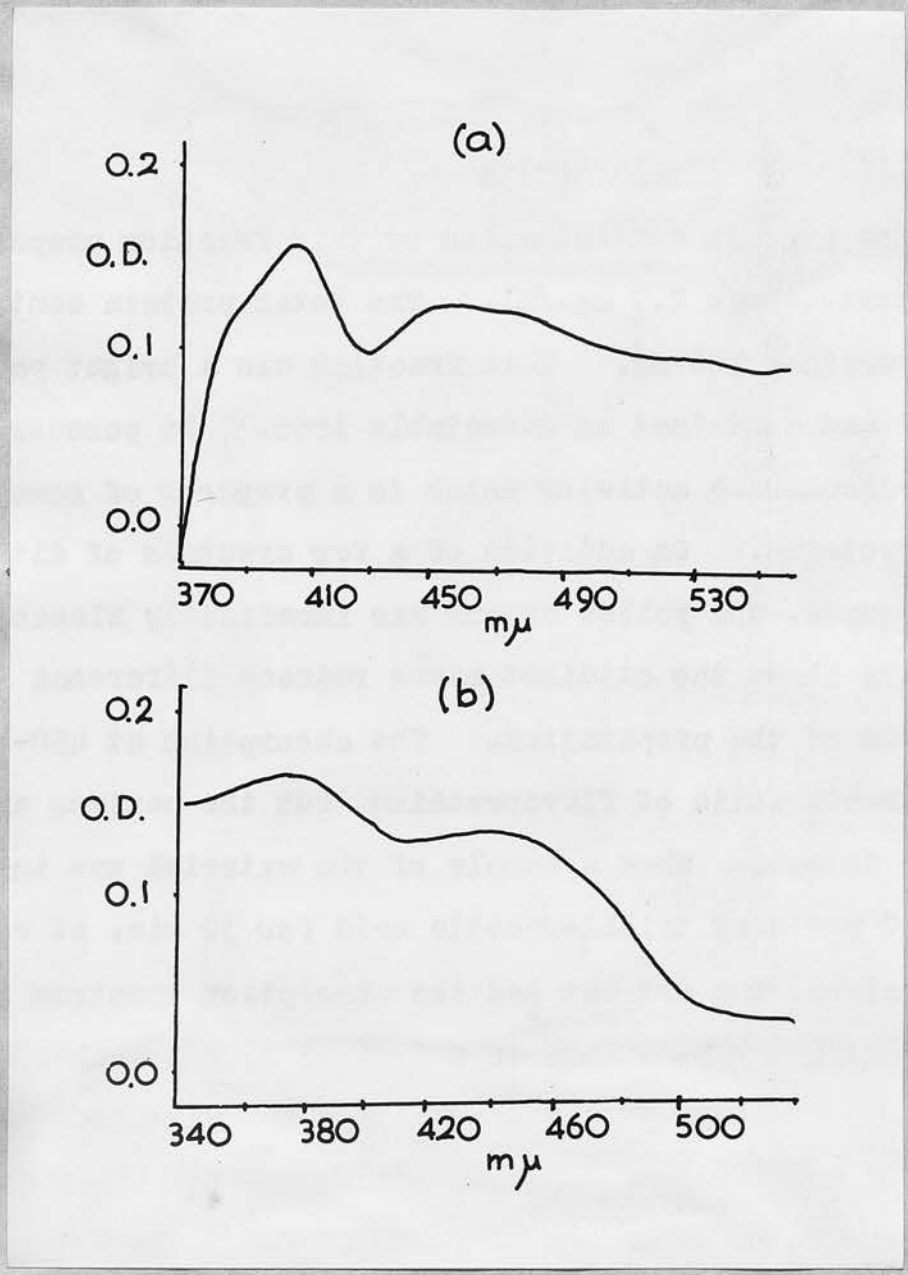


Fig.49.(a) Oxidised minus dithionite-reduced difference spectrum of NADPH-diaphorase.

(b) Absorption spectrum of prosthetic group of NADPH-diaphorase extracted with 10% trichloracetic acid.

(ii) NADPH-diaphorase

The protein concentration of this fraction prepared as described was 2.7 mg./ml. The total protein content was therefore 108 mg. This fraction was a bright yellow colour and contained no detectable iron. It possessed NADPH-diaphorase activity which is a property of some flavoproteins. On addition of a few crystals of dithionite to a sample, the yellow colour was immediately bleached. Fig. 49a shows the oxidised minus reduced difference spectrum of the preparation. The absorption at 450-490 mμ is characteristic of flavoproteins, but the maximum at 410 mμ is not. When a sample of the material was incubated with 10 per cent trichloracetic acid for 30 min. at room temperature, the extract had the absorption spectrum shown in fig. 49b. This is a typical flavin nucleotide spectrum. The fluorescence spectrum was also investigated. Using an excitation wavelength of 465 mμ, the fluorescence spectrum showed a maximum at 520 mμ. When the emission was held at 520 mμ, the excitation beam showed a maximum at 460-465 mμ. This again is typical of a flavin nucleotide. A sample of the diaphorase was dialysed overnight against distilled water. The dialysed material was extracted with 10 per cent trichloracetic acid as above, and the extract shaken with ether five times, to remove trichloracetic acid. The resulting salt and acid-free extract was spotted on a paper chromatogram along with standard FAD, FMN and riboflavin. The chromatogram

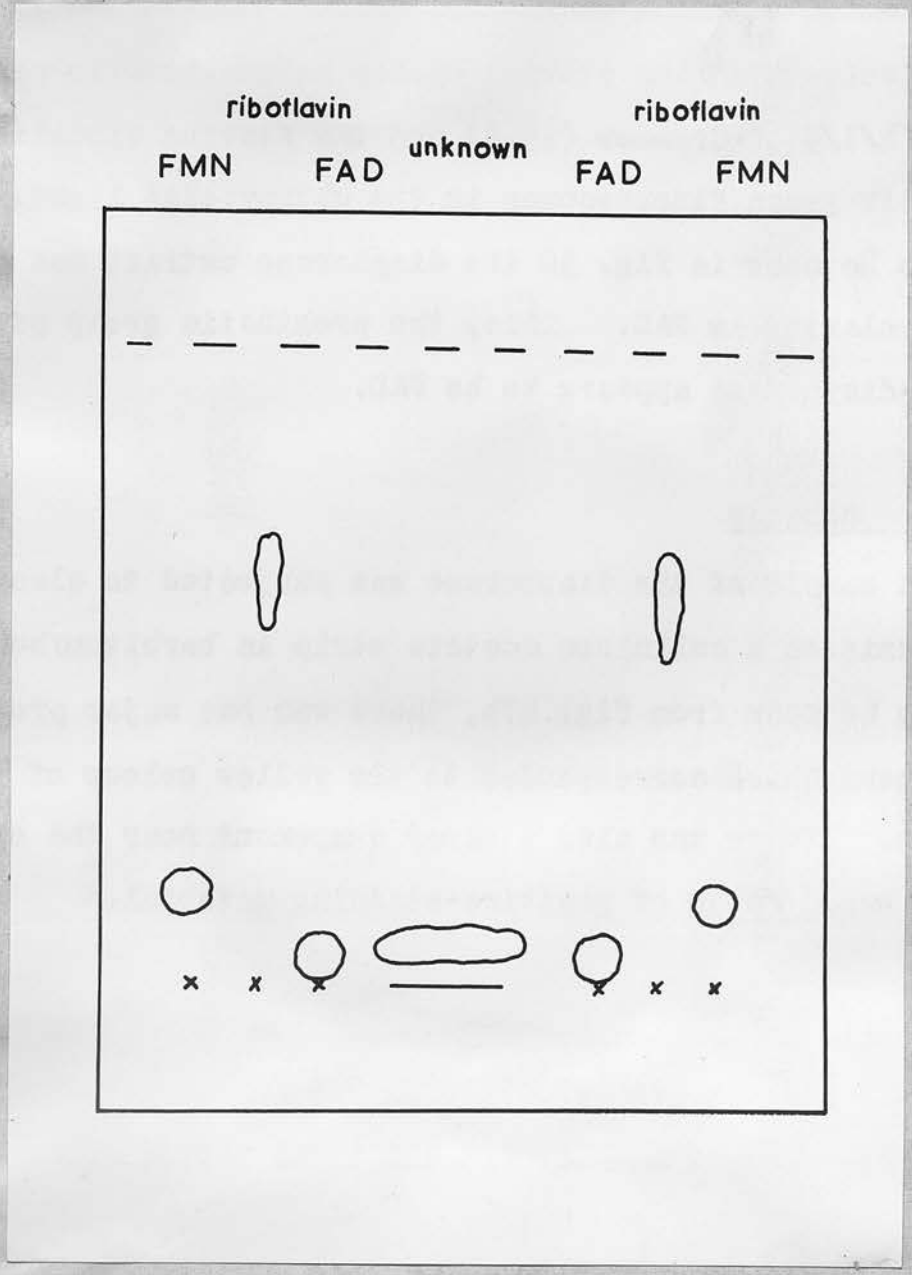


Fig. 50. Paper chromatogram of the prosthetic group of the NADPH-diaphorase in the solvent system butanol/acetic acid/water 4:1:5, along with standard FAD, FMN, and riboflavin.

was developed in the solvent system butanol:acetic acid: water 4/1/5 of Crammer (1948) and the flavins visualised by their green fluorescence in the ultraviolet light. As can be seen in fig. 50 the diaphorase extract had the same polarity as FAD. Thus, the prosthetic group of the NADPH-diaphorase appears to be FAD.

Electrophoresis

A sample of the diaphorase was subjected to electro-:phoresis on a cellulose acetate strip in barbitone buffer. As can be seen from fig. 47b, there was one major protein component which corresponded to the yellow colour of the flavin. There was also a minor component near the origin and a broad smear of positive-staining material.

(iii) Cytochrome P-450

The protein concentration in the sample prepared as described was 2.8 mg./ml. and the total protein content was therefore 112 mg. When the fractions of this component were removed from the G-200 column, they were observed to be cloudy in appearance. On sonication at 0°C for 10 min., clarification was observed, leaving a red-brown solution only faintly opalescent. On storing frozen overnight and thawing, the opacity reappeared, but could be removed by sonication once more. Storing at 0°C for two days also resulted in a gradual increase in opacity. Clarification could again be achieved by

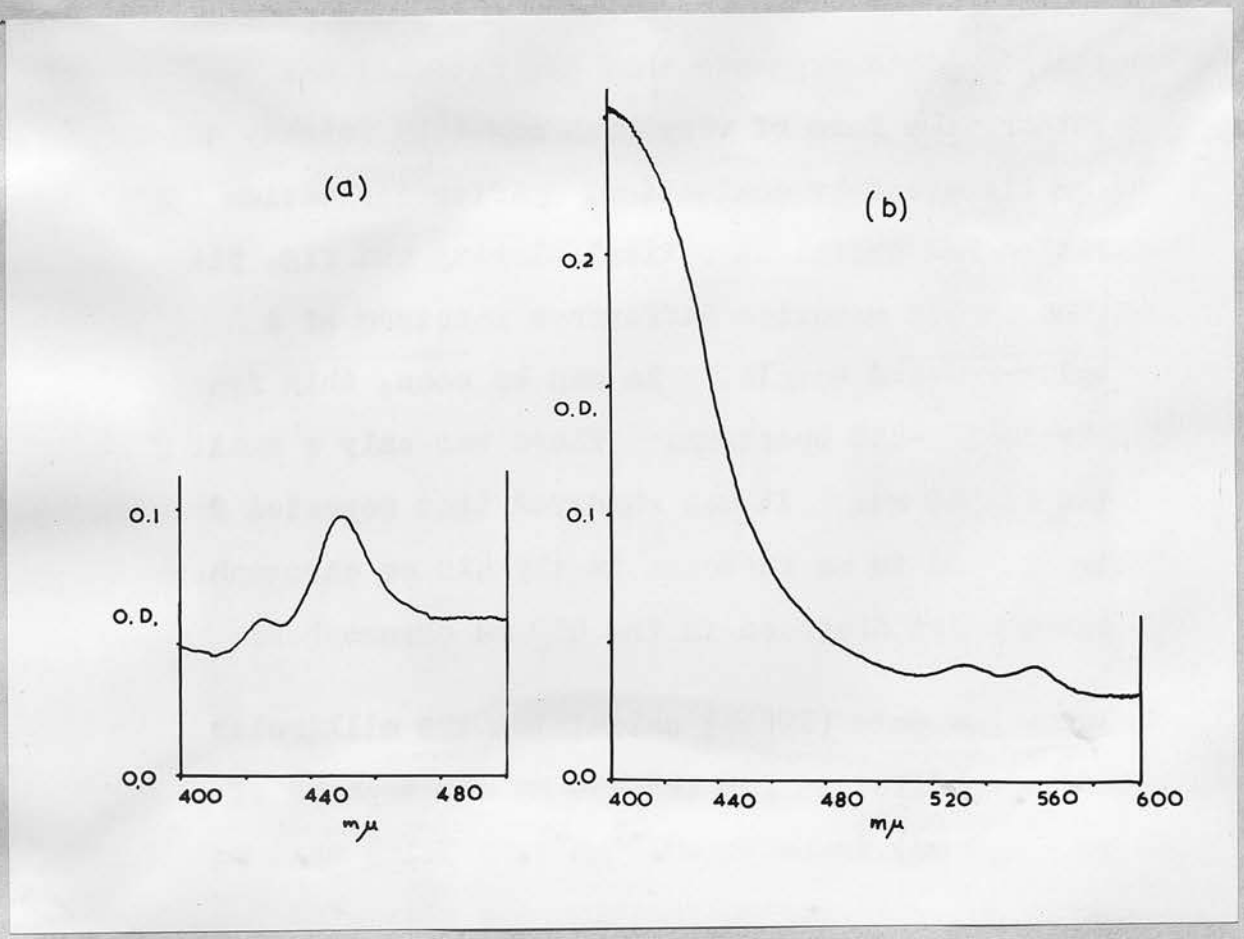


Fig. 51(a). Dithionite-reduced carbon monoxide difference spectrum of fractionated cytochrome P-450. Protein content 1.4mg./ml.

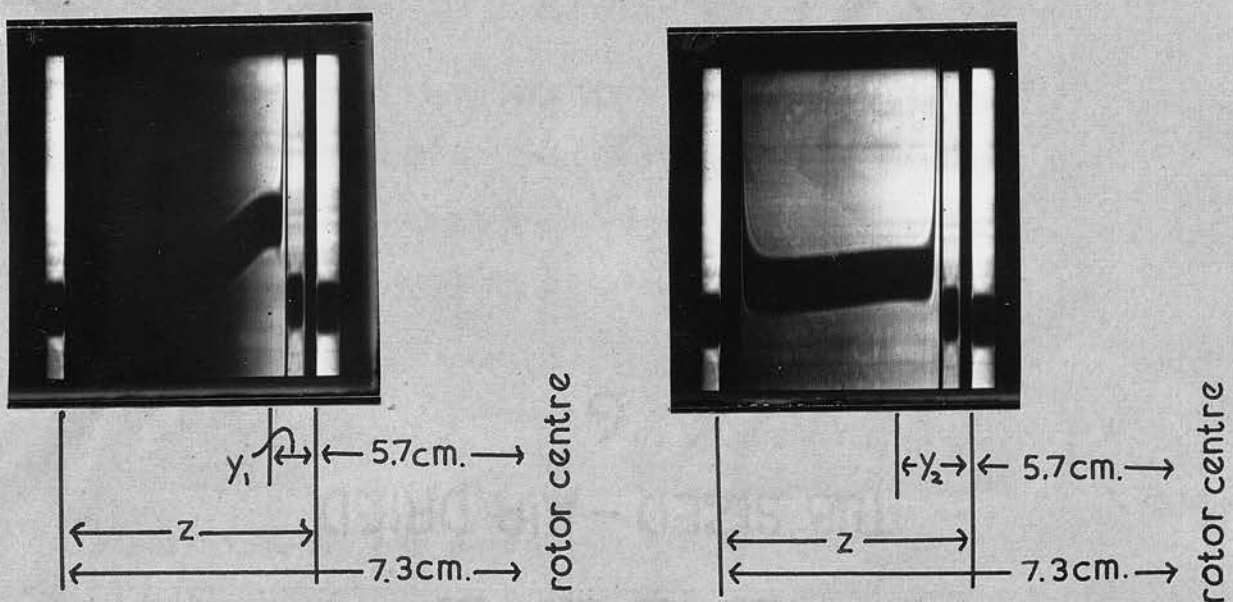
(b) Pyridine haemochromogen of acid acetone extract of cytochrome P-450 fraction. Reduced with dithionite.

sonication. This suggests that the material was aggregating to a form of very high particle weight, which could be dispersed by sonication. After sonication the preparation had excellent optical clarity and fig. 51a shows the carbon monoxide difference spectrum of a dithionite-reduced sample. As can be seen, this fraction had a typical P-450 spectrum. There was only a small shoulder at 420 mμ. It was observed that repeated freezing and thawing led to an increase in the 420 mμ chromophore with concomitant decrease in the 450 mμ chromophore.

Omura and Sato (1964b) calculated the millimolar extinction coefficient for the 450 mμ chromophore of P-450 (450 mμ - 490 mμ) to be 91 $cm.^{-1}mM^{-1}$. Using this value, the amount of P-450 in the fraction was estimated from the height of the 450 mμ chromophore of fig. 51a and found to be -

1 mole P-450/2.9 x 10^6 g. protein.

The amount of haem in the fraction was also estimated by the pyridine haemochromogen method (Morrison and Horie, 1965). To 2 ml. of the sample was added 0.5 ml. 25 per cent trichloracetic acid. The precipitate was homogenised twice with 3 ml. acid acetone (1.8 ml. conc. hydrochloric acid/100 ml. acetone). To 5 ml. extract was added 7.5 ml. pyridine and 1 ml. IN sodium hydroxide. The absorption spectrum was recorded in a cell of 3 cm. light path after addition of a spatula tip of dithionite and is shown on



y_1 - 0.57cm. y_2 - 0.95cm. z - 3.4cm.

Fig.52. Schlieren pattern of cytochrome P-450 showing the parameters for calculation of the sedimentation constant.

fig. 51b. Taking the millimolar extinction coefficient of the pyridine haemochromogen of protohaem to be $34.2 cm.^{-1} mM.^{-1}$ at 557 mμ (Morton, 1958), the amount of haem in the cytochrome P-450 fraction was found to be -

$$1 \text{ mole haem} / 3.1 \times 10^6 \text{ g. protein,}$$

which agrees quite well with the amount of P-450. Thus, P-450 accounted for all the haem in the preparation. Also, unless the sample was very impure, the haem group was bound to a protein species of very high particle weight, even after sonication.

Ultracentrifugal analysis

A sample of the P-450 fraction was concentrated with 'Carbowax' to a concentration of 10 mg./ml. and the sedimentation properties studied using a Spinco model L analytical ultracentrifuge. The sample was sonicated for 10 min. at 0°C immediately before the experiment, but this concentrated preparation was still somewhat opaque after this treatment. The centrifugation run was conducted at a rotor speed of 33,100 r.p.m., and a temperature of 12.7°C. Fig. 52 shows the Schlieren pattern at two stages during the experiment, 416 sec. apart. It can be seen that as the protein separated from the meniscus it did not form a sharp peak but rapidly spread out into a diffuse band. This indicates that a broad spectrum of particle weights was present in keeping with the aggregation phenomenon mentioned above.

It is also evident that the unsedimented preparation was opaque to the light of the mercury vapour lamp. However, the very dense material apparently sedimented very rapidly leaving a slower-moving, less dense component. It is suggested that the latter was haem-containing material. Haem prosthetic groups would be expected to absorb some of the light from the mercury vapour lamp, particularly the bands of shorter wavelength. The former material would then consist of the very highly aggregated components. An attempt was made to calculate the sedimentation constant for the material at the height of the protein peak. This could only be done very approximately for the following reasons:-

a) the protein band spread out rapidly and did not form a sharp peak.

b) it was only possible to take two photographs before the band spread throughout the whole cell.

c) it was not possible to measure the sedimentation constant as a function of protein concentration because increasing the protein concentration would have resulted in a further deterioration of the optical properties of the material, and decreasing the protein concentration would have made it even more difficult to assign a position to the protein peak maximum. This maximum agreed tolerably well with the turning point of the change in optical density assumed to be due to the haem group. The

Table 11

Calculation of Sedimentation Constant for P-450

Speed of rotor 33,100 r.p.m.

Bar angle 45°

Temperature $12.7^{\circ}C$

Odiometer Readings

1st photograph	684536
2nd photograph	684572
difference	36

1 odiometer unit = $\frac{16 \times 59,780}{149.5}$ rotor revs.

$\therefore$ time between photographs = $\frac{36 \times 16 \times 59,780}{33,100 \times 149.5}$ secs. = 416 secs.

distance of protein peak from rotor centre

$$x_1 = y_1 \times \frac{1.6}{2} + 5.70 = 5.98 \quad \therefore \ln x_1 = 1.7884$$

$$x_2 = y_2 \times \frac{1.6}{2} + 5.70 = 6.17 \quad \therefore \ln x_2 = 1.8197$$

$$\therefore \ln x_2 - \ln x_1 = 0.0313$$

sedimentation constant

$$s = \frac{d(\ln x)}{dt} \cdot \frac{1}{\omega^2} \quad \text{where } \omega^2 = (2\pi \times \frac{\text{r.p.m.}}{60})^2$$

$$\therefore s \simeq \frac{0.0303}{416} \frac{10^{-7}}{1.205} = 62.5 \times 10^{-12}$$

$\therefore$ sedimentation constant $\simeq$ 63S

sedimentation constant was calculated as in Table 11 and came to 63S. A rough estimate of the particle weight was obtained from the relationship -

$$\frac{S_1}{S_2} = \left(\frac{MW_1}{MW_2}\right)^{\frac{2}{3}}$$

where S_1 and MW_1 are the sedimentation constant and molecular weight of the unknown protein and S_2 and MW_2 are the sedimentation constant and molecular weight of a known protein. In this case the known protein chosen was sheep thyroglobulin with a sedimentation constant of 19S and a molecular weight of 660,000 (Salvatore, Salvatore, Cahnmann and Robbins, 1964). Substituting these values in the above formula, the particle weight of the P-450 fraction at the peak maximum came to 4×10^6, which is in surprisingly good agreement with the value of 3×10^6 calculated from the haem content. If this does represent the particle weight of the non-aggregated cytochrome P-450 preparation it is probably the smallest particle to which this cytochrome is bound yet reported, although still very large.

Electrophoresis

The material was subjected to electrophoresis on a cellulose acetate strip in barbitone buffer as shown in fig. 47c. As can be seen, after 3 hr., the P-450 still

remained firmly bound to the origin. There appeared, however, to be a smear of material running away from the origin, which could possibly have contained contaminating NADPH-diaphorase.

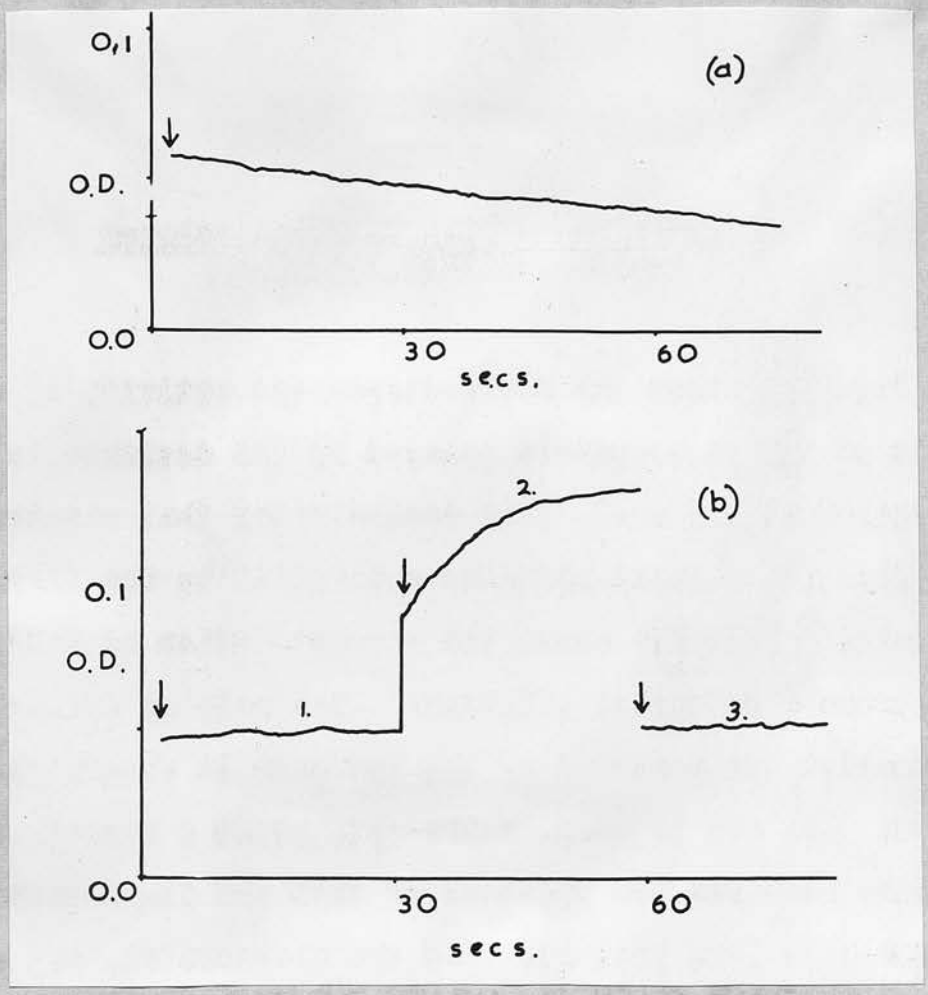


Fig.53(a) Bleach of 590mμ chromophore of dye effected by NADPH-diaphorase in the presence of an NADPH-generating system. Both cells contained the dye in phosphate buffer. A sample of diaphorase was added to the experimental cell to initiate the reaction.

(b) NADPH - cytochrome c reductase activity. Both cells contained phosphate buffer and 0.3mg. cytochrome c. The solutions were scanned at a fixed wavelength of 550mμ.

1. Addition of 0.46mg. diaphorase to experimental cell.
2. Addition of 0.46mg. diaphorase and 0.40mg. non-haem iron protein to experimental cell.
3. Addition of 0.40mg. non-haem iron protein to experimental cell.

(7) Reconstitution of the NADPH-cytochrome P-450 reductase system

Fig. 53a shows the NADPH-diaphorase activity of a sample of the flavoprotein assayed by the decrease in absorption at 590 mμ. This demonstrates that electrons pass from the reduced pyridine nucleotide to the flavo-:protein. Fig. 53b shows the reconstitution of NADPH-cytochrome c reductase activity. The rate of cytochrome c reduction was measured by the increase in absorption at 550 mμ. As can be seen, NADPH-cytochrome c reductase activity required the presence of both the diaphorase and the non-haem iron protein. As the flavoprotein can accept electrons directly from NADPH, it appears likely that in the reduction of cytochrome c, electrons were passing from the reduced pyridine nucleotide to the flavoprotein, and then to the non-haem iron protein, and finally to the cytochrome.

That this is the most likely pathway of electron flow is confirmed in fig. 54a which shows that the reduction of the non-haem iron protein, as manifest by a bleaching of the chromophores at 414 and 455 mμ, was achieved by NADPH only in the presence of the flavoprotein, although it could be reduced directly by dithionite.

Finally, in fig. 54b is shown the reconstitution of NADPH-cytochrome P-450 reductase activity. (1) Is the

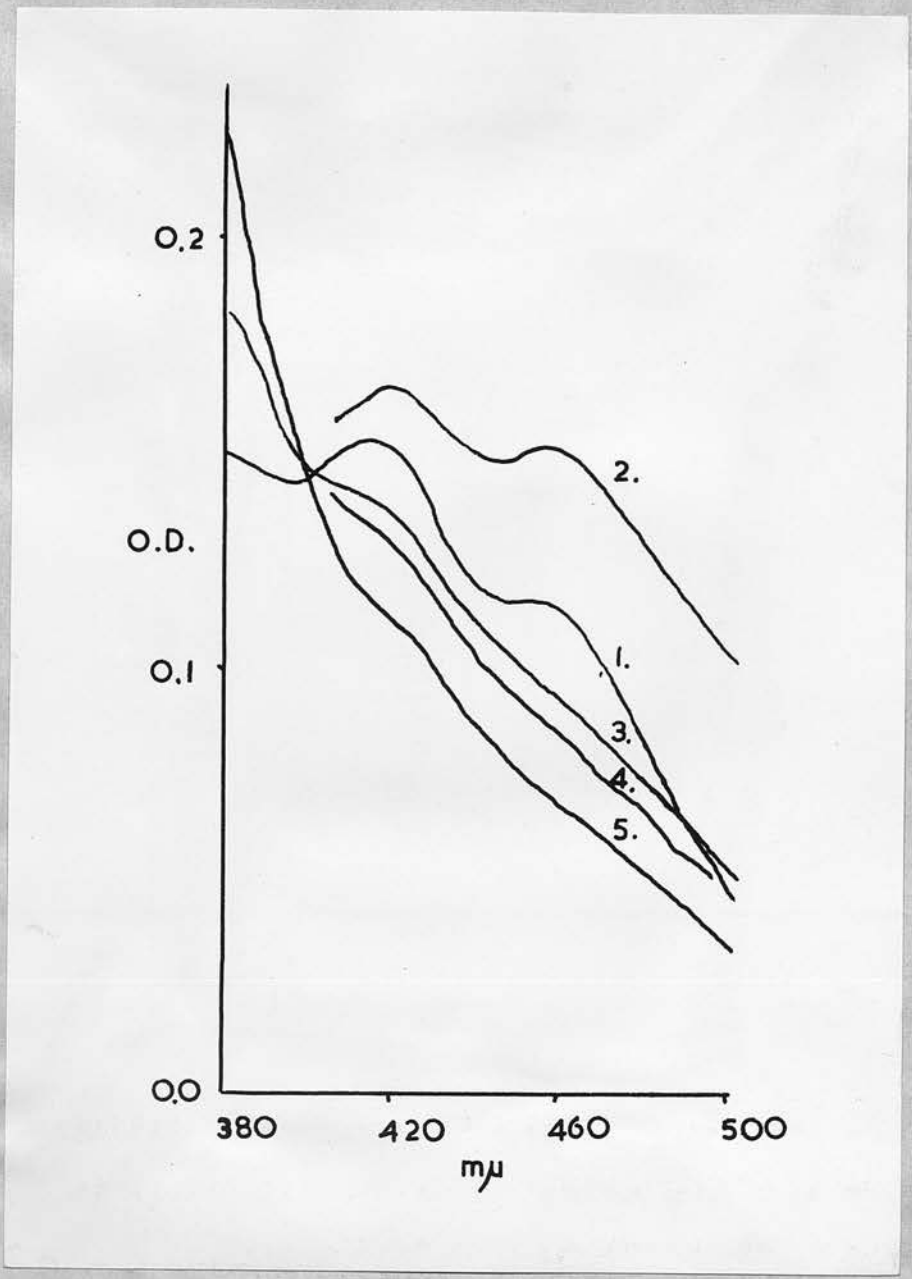


Fig.54(a). Reduction of non-haem iron protein.

1. Absorption spectrum of non-haem iron protein in phosphate buffer pH7.4. (2.0mg. protein)
2. Addition of NADPH-generating system to both cells.
3. As (2). with addition of 0.46mg. diaphorase to experimental cell. Scanned immediately.
4. As (3). Scanned 15min. later.
5. As (1). A few crystals dithionite added to experimental cell.

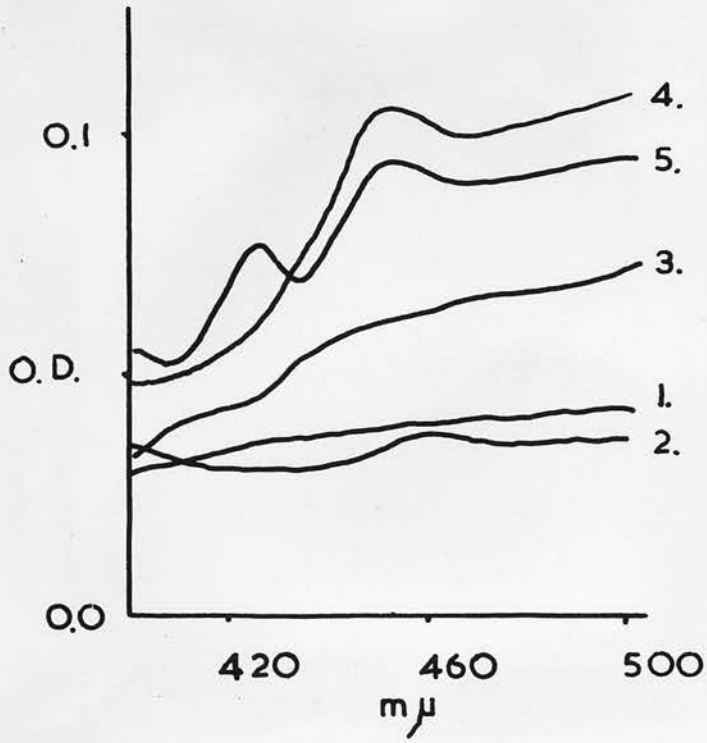


Fig.54(b). NADPH-cytochrome P-450 reductase activity.

1. Both cells - 1.5mg. cytochrome P-450 fraction in phosphate buffer pH7.4, plus NADPH-generating system.
2. As (1), with experimental cell gassed with carbon monoxide 30sec.
3. As (2), with 0.2mg. diaphorase added to experimental cell.
4. As (3), with 0.1mg. non-haem iron protein added to experimental cell.
5. As (4), with few crystals dithionite added to experimental cell.

baseline with both cells containing P-450 plus NADPH-generator. When the experimental cell was gassed with carbon monoxide, there was no change in absorption as shown in (2). Addition of the diaphorase also did not change the spectrum as shown in (3). However, a final addition of the non-haem iron protein did give the characteristic 450 mμ absorption of P-450 (4). Thus, for reduction of P-450 by NADPH, both the flavoprotein and the non-haem iron protein are required. As the sequence of electron flow has been shown to be - NADPH → flavoprotein → non-haem iron protein, this means that electrons from the NADPH pass along this chain in order to reduce P-450. As three fractions, namely the flavoprotein, the non-haem iron protein and the P-450 fraction are required for cholesterol side-chain cleavage activity, this perhaps suggests that the same electron transport chain is required to provide reducing equivalents for the side-chain cleavage of cholesterol. Fig. 55 shows the scheme proposed therefore for the cholesterol side-chain cleavage system. In this, electrons pass from the reduced pyridine nucleotide via the flavoprotein and the non-haem iron protein to the P-450 which contains sites for binding and/or activation of both substrate and molecular oxygen. This scheme is similar to that proposed by Omura et al (1966) for the steroid 11β-hydroxylase (fig. 11).

Fig. 54b shows that when P-450 was reduced by the

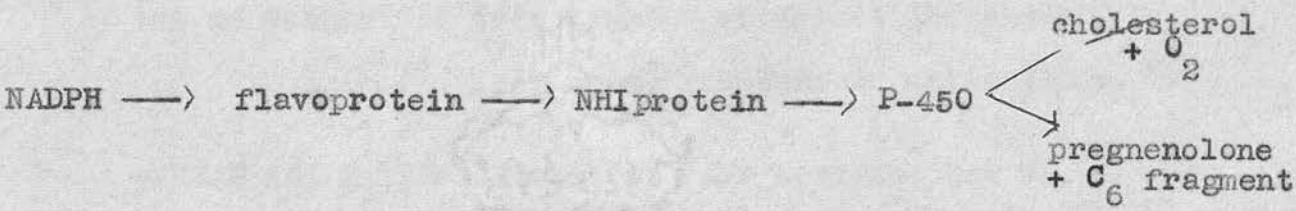


Fig.55. Proposed scheme of electron flow in the cholesterol side-chain cleavage system.

NHI - non-haem iron.

reductase system, there was no absorption band at 420 mμ (4). On addition of dithionite however, a small 420 mμ chromophore appeared (5). As haemoglobin is reduced by the reductase system to give a chromophore at 420 mμ in the presence of carbon monoxide, this means that the 420 mμ chromophore in fig. 54a was not due to contaminating haemoglobin but was due entirely to P-420, and P-420 was not reduced by the reductase system. Thus, the chromophore at 420 mμ observed in earlier preparations such as mitochondrial sonicates reduced with NADPH must be due to contaminating haemoglobin (e.g. fig. 18).

It was observed that in reconstituting the NADPH-cytochrome P-450 reductase activity, the chromophore at 450 mμ could be produced in the presence of the non-haem iron protein without added diaphorase. This means that in the time taken to mix the samples and record the spectrum, there was sufficient flavoprotein contaminating the P-450 preparation to effect its reduction. Unfortunately it was not possible to carry out a proper time-course study of cytochrome P-450 reduction as it was in the case of cytochrome c (fig. 53b), because the changes in optical density were too small to be recorded without a dual-wavelength spectrophotometer in which the optical density at 450 mμ could be compared directly with that of any other wavelength, such as 490 mμ.

(8) Summary

(a) Attempts were made to fractionate the cholesterol side-chain cleavage system using ammonium sulphate. These were unsuccessful.

(b) Prolonged ultracentrifugation of a 105,000 xg.-30 min. supernatant of mitochondrial sonicate gave a pellet containing P-450 and a clear supernatant. The pellet contained no side-chain cleavage activity and the supernatant contained about one-third of the activity of the recombined supernatant plus pellet, although it contained no detectable P-450.

(c) Chromatography of the 105,000 xg.-30 min. supernatant of mitochondrial sonicate on Sephadex G-100 gave two fractions corresponding to those obtained by centrifugation above. Again, both fractions were required for maximum activity, but this time the fraction containing the P-450 possessed some activity of itself.

(d) Chromatography of acetone powder extracts on Sephadex G-200 and DEAE Sephadex A-25, yielded three components, namely an NADPH-diaphorase, a non-haem iron protein, and a fraction containing P-450. The former two constitute an NADPH-cytochrome P-450 reductase. These three components were necessary to reconstitute the cholesterol side-chain cleavage system.

(e) Some of the chemical and physical properties of these three protein fractions are described.

8. DISCUSSION

In the General Introduction it was pointed out that studies on the cholesterol side-chain cleavage system were hampered by lack of a suitable assay. The Michaelis constant for the reaction is very low (2 μM) which means that the products of the reaction are present in very small quantities. In purified preparations the sole product is pregnenolone, and this has no chromophore which is readily detectable by absorption or fluorescence spectro-:metry.

These problems were largely overcome by the use of ^{14}C-labelled substrate of high specific activity and the development of T.L.C. solvent systems consisting of petroleum, acetic acid and an ether, which give good separation of the products from one another and from cholesterol. Radiochromatogram scanning and liquid scintillation spectrometry were the techniques used to detect and quantitate the products. Using this method a large number of analyses could be performed in a few hours, making it possible to undertake kinetic studies which would have been impossible using more time-consuming and laborious methods. Moreover, the sensitivity of the method made it possible to investigate the presence of metabolites which might be present in only trace amounts.

Using this assay method, a study of the subcellular localisation of cholesterol side-chain cleavage activity in bovine adrenal cortex revealed that the activity resided in the mitochondria, an observation which is in

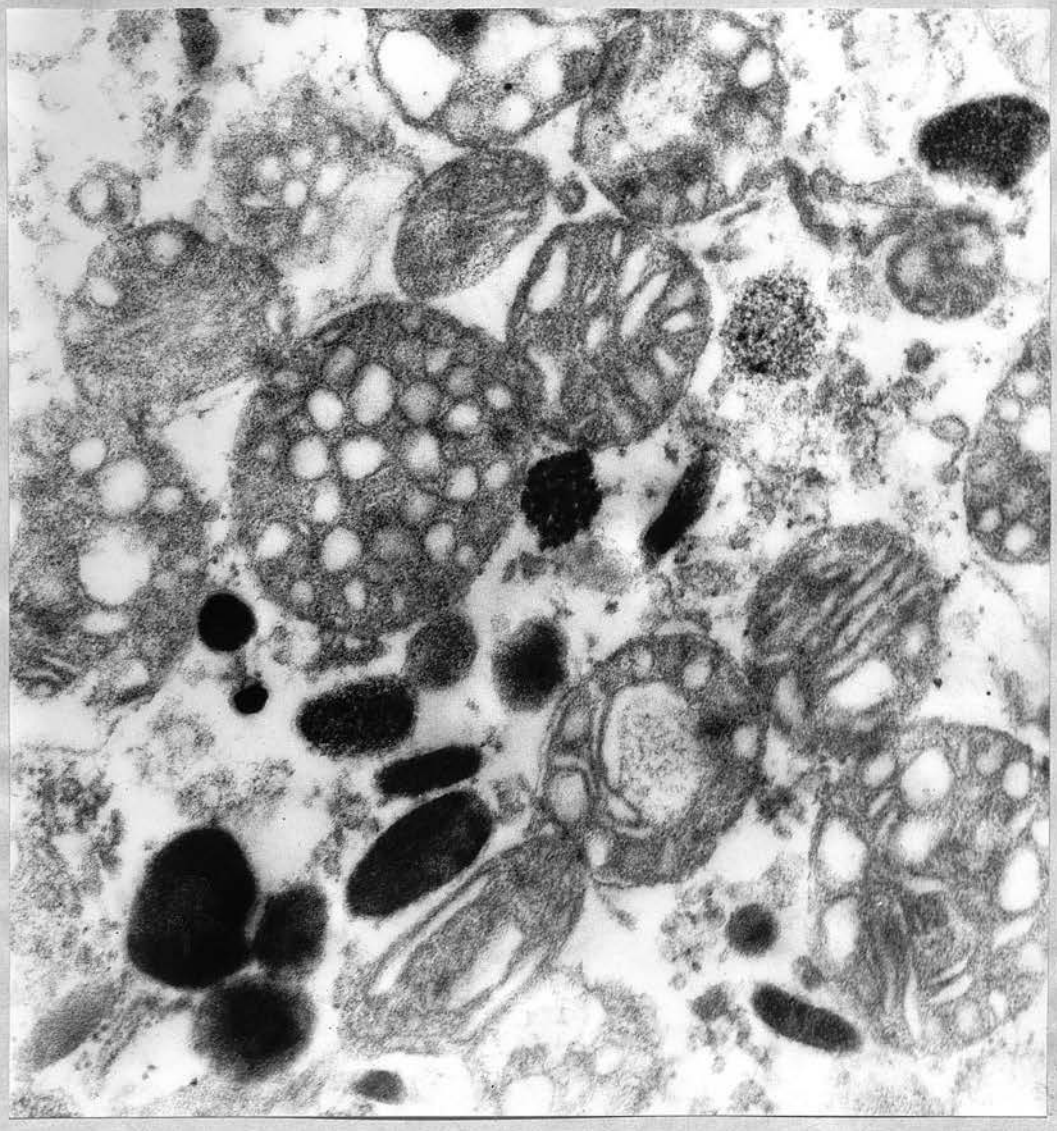


Fig.56. Electron micrograph of mitochondrial pellet prepared as described in chapter 2.
Fixed with Dalton's fixative and stained with uranyl acetate and lead acetate. Magnification 30,000.
The field is largely occupied with mitochondria which are somewhat swollen. The dense objects are lysosomes. The occasional small circular objects are microsomes.

keeping with earlier reports. However, in mitochondria isolated as described in Chapter 2, the pregnenolone formed as a result of the cleavage reaction was metabolised to a range of products including progesterone, 21-hydroxy-:progesterone (deoxycorticosterone) and more polar steroids. This again is in keeping with other reports, e.g. Halkerston et al (1961), but is contrary to the generally-held view that the enzymes responsible for the further metabolism of pregnenolone, namely the 3β-ol dehydrogenase and the Δ^5-3-ketosteroid isomerase, are located in the endoplasmic reticulum.

Fig. 56 shows an electron micrograph of a sample of the twice-washed mitochondrial pellet. The mitochondria are somewhat swollen, as would be expected of mitochondria isolated in 0.25M sucrose (Green and Fleischer, 1960). Also present are some fragmented mitochondria and densely-staining lysosomes, but few microsomes. It seems unlikely therefore that the further metabolism of pregnenolone is due entirely to contamination by microsomal enzymes. This is in agreement with the suggestion of Koide and Torres (1965) of a mitochondrial 3β-ol dehydrogenase and Δ^5-3-ketosteroid isomerase. However, the formation of 21-hydroxyprogesterone does imply a certain degree of contamination.

In attempts to solubilise the enzyme system from the mitochondria, it was found that the cholesterol side-chain

cleavage activity could be separated from the bulk of the mitochondrial protein either by subjecting the mitochondria to ultrasonication followed by centrifugation, or by extracting an acetone-dried preparation of mitochondria with phosphate buffer followed by centrifugation. In both cases, the cholesterol side-chain cleavage activity remained in the supernatant obtained after the centri-:fugation step. In such preparations, in contrast to intact mitochondria, pregnenolone was virtually the only product formed, indicating that the 3β-ol dehydrogenase and/or the Δ^5-3-ketosteroid isomerase remained bound to the sedimented fraction.

It was also found in these 'solubilised' preparations that the cholesterol side-chain cleavage activity was many times greater than in native mitochondria, on a tissue wet-weight basis. This could have been due to inaccessibility of the cleavage enzymes in native mito-:chondria to the pyridine nucleotide and/or the cholesterol-4-^{14}C substrate. It is known that pyridine nucleotides do not readily penetrate mitochondrial membranes. In this connection it is worthy of note that succinate and other citric acid cycle intermediates were found by Koritz (1966) to be better donors of electrons to the cholesterol side-chain cleavage system in native mito-:chondria than was exogenous NADPH.

It is possible that, in native mitochondria, the radioactive cholesterol substrate is not readily available to the enzyme system due to equilibration with the endogenous mitochondrial cholesterol pool, which would consequently dilute it to an unknown extent, or due to inaccessibility of the enzymes to exogenous cholesterol. These conditions would have the effect of raising the value of the experimentally determined apparent Michaelis constant for the reaction. Raggatt and Whitehouse (1966) found that the apparent Km. in native mitochondria was 50 µM, whereas we have found the Km. using supernatant of mitochondrial sonicate and acetone powder extracts, which contain very little endogenous cholesterol, to be 2-4 µM. If the radioactive cholesterol is indeed equilibrating with endogenous cholesterol in native mitochondria, it may be that the precursor pool of substrate for the side-chain cleavage reaction in vivo is mitochondrial cholesterol.

Because the assay method lent itself to the investigation of very small amounts of metabolites, attempts were made to detect 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol in incubations with cholesterol-4-^{14}C. In a study of the early events in side-chain cleavage, a lag phase in the formation of pregnenolone was observed in incubations performed at 20°C, suggesting that an intermediate was being formed prior to the formation of pregnenolone. Yet the only detectable metabolite was pregnenolone. This means that, if an intermediate is

present, (a) it was not extracted from the protein by the extraction procedure using boiling ethyl acetate, or (b) the rate of breakdown of the intermediate to pregnen-:olone relative to its rate of formation from cholesterol was so rapid as to render its concentration below the limit of sensitivity of the assay which was estimated to be 5×10^{-4} µM. or 100 counts/min. This is about 0.03 per cent of the radioactivity added to an incubation, and is at least 4000 times less than the Km. To test the former possibility, protein residues from incubations extracted in the normal manner were exhaustively re-extracted with boiling ethyl acetate. This succeeded in extracting only cholesterol and pregnenolone. The protein residue from this extraction procedure was combusted and the carbon dioxide formed was trapped in Hyamine and the radioactivity determined. This revealed that only 115 counts/min. per incubation remained in the protein residue in this way. There was no reason to suppose that this radioactivity was due to anything other than a residual amount of cholesterol and pregnenolone. Hence no evidence was obtained for the existence of a protein bound inter-:mediate which was not extracted with ethyl acetate.

Much of the work presented here is concerned with providing evidence that cytochrome P-450 is the active centre of the cholesterol side-chain cleavage system. If this is so, the concentration of P-450 in a standard incubation should provide some idea of the order of

magnitude of the enzyme-substrate complex. This was calculated from the difference in optical density at 450 mμ minus 480 mμ in the reduced carbon monoxide difference spectrum, using the millimolar extinction coefficient of 91mM.$^{-1}$cm.$^{-1}$ quoted by Omura and Sato (1964). The concentration of P-450 in a standard incubation calculated in this way was 0.1 μM. The concentration of enzyme-substrate complex present will only be a fraction of this, as the active centre is only about half-saturated with substrate under the conditions of standard assay (the Km. is 2.0 μM. and each incubation contained 6.8 x 10^{-3} μ moles cholesterol-4-^{14}C in 5 ml., i.e. a concentration of 1.4 μM.). Also, possibly only a fraction of the total P-450 present is involved in cholesterol side-chain cleavage, as will be discussed later. However, this concentration of 0.1 μM. is 200 times greater than the sensitivity limit of the assay (5 x 10^{-4} μM). Hence the concentration of any intermediate present is likely to be only a small percentage of the concentration of enzyme-substrate complex, which must therefore consist almost entirely of cholesterol and pregnenolone.

These experiments do not rule out the presence of 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol as intermediates, but they do virtually rule out the existence of separate sites for 20α- and 22 -hydroxylation, as this would require that 20α-hydroxycholesterol dissociate

from the first hydroxylation site and accumulate in the solution before becoming bound to the second site. Thus the sequence of reactions from cholesterol to pregnenolone may take place at one binding site.

Attempts were then made to accumulate intermediates in the formation of pregnenolone from cholesterol in the presence of unlabelled 20α-hydroxycholesterol or pregnen-:olone. Again, no radioactive 20α-hydroxycholesterol could be demonstrated, in agreement with the findings of Hall and Kortiz (1964).

However, other derivatives of cholesterol hydroxylated in the side-chain, namely 24-,25- and 26-hydroxycholesterols, were effective inhibitors as well as 20α-hydroxycholesterol. Moreover, inhibition by 20α-hydroxycholesterol and these other derivatives was competitive with respect to cholesterol. Previous studies on the inhibition of side-chain cleavage by 20α-hydroxycholesterol showed this inhibition to be non-competitive with respect to cholesterol (Hall and Koritz, 1964; Raggatt and Whitehouse, 1966). This suggested that the added 20α-hydroxycholesterol was occupying a binding site other than that occupied by cholesterol, presumably the site of 22-hydroxylation. That the existence of separate hydroxylation sites is unlikely has been discussed in the previous paragraph. Our evidence that this inhibition by hydroxycholesterol derivatives is competitive with respect to cholesterol supports the concept of a single reaction site, and suggests that these derivatives

including 20α-hydroxycholesterol are simply competing with cholesterol for this site. Thus we have been unable to obtain any information to suggest that the inhibition of cholesterol side-chain cleavage by 20α-hydroxycholesterol is such as to give it special significance as a possible intermediate between cholesterol and pregnenolone.

The strongest remaining evidence for the involvement of 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol as intermediates in the reaction is therefore the fact that these are claimed to be more efficient precursors of pregnenolone than is cholesterol. However, the same has been shown to be true for 22-hydroxycholesterol (Chaudhuri et al, 1962), while 20α-hydroxy-22-ketocholesterol is also metabolised to pregnenolone, but has been ruled out as an obligatory intermediate (Constantopoulos et al, 1966). Thus if ability to be metabolised to pregnenolone is accepted as evidence for involvement as an intermediate, it is necessary to assume several possible pathways between cholesterol and pregnenolone, which seems somewhat unlikely.

Thus these experiments suggest that the cleavage of the cholesterol side-chain to form pregnenolone is a concerted reaction occurring at one enzyme site. Under suitable conditions a lag phase in the formation of pregnenolone can be detected indicative of one or more intermediates, but all attempts to detect these have failed. Thus, any intermediate must have a very transient existence suggesting that the initial oxidative attack on

the cholesterol molecule is the rate-limiting one. The involvement of 20α-hydroxycholesterol and 20α,22ξ-dihydroxy-:cholesterol as intermediates has not been ruled out, but their average life-span must be so short that they have virtually no independent existence. In the limiting case, the side-chain cleavage would occur as a concerted series of reactions with no free intermediates. Oxygen and cholesterol would be bound to the enzyme surface in a transition-state complex and the steroid would remain bound until the sequence of reactions leading to liberation of the side-chain was completed. A reaction scheme on these lines is discussed later.

Any reaction mechanism proposed for the cholesterol side-chain cleavage reaction must take into account the metabolite, as yet unidentified, isolated in trace amounts from incubations with cholesterol-^{14}C. This did not appear before pregnenolone in a time-course study, but gradually accumulated with time, suggesting it might be a side-product of the reaction. It is a C_{27} steroid, and its polarity in the solvent system A and B suggests it might be a cholesterol derivative with an oxygen function in the side-chain (cholesterol derivates with oxygen functions in the ring structure are much more polar, e.g. 7-oxo cholesterol). Also, it is less polar than 20α-hydroxycholesterol (itself the least polar of the side-chain-hydroxycholesterol derivatives), suggesting this oxygen function to be a ketone, a hydroperoxide, or

an epoxide. The likelihood of this was strengthened by the fact that treatment with lithium aluminium hydride gave a more polar product suggesting reduction of one of the above to a hydroxyl. The polarity of this reduction product coincided with that of 22-hydroxycholesterol. Unfortunately diagnostic tests for ketones, hydroperoxides and epoxides all proved negative.

Incubation of this metabolite with an acetone powder extract in the presence of an NADPH-generator produced a compound with the polarity of pregnenolone, but the rate of conversion was less than that of cholesterol in a parallel incubation, again suggesting the unknown metabolite is not a direct intermediate in the cleavage of the cholesterol side-chain.

The structure of this unknown metabolite has not yet been established. The work of identification was greatly hindered by the fact that the metabolite was present in only nanogram quantities, making it impossible to apply standard techniques of analysis such as infra red and nuclear magnetic resonance spectroscopy. The only way the problem could be approached was to make derivatives and compare their polarities on T.L.C. with those of authentic compounds. However, the possibility that the compound might be a 20,22-oxidocholesterol seems attractive in view of recent evidence for the formation of epoxides as intermediates or side-products in mixed-function oxidation reactions (e.g. Corey et al, 1966;

Change and Sih, 1964), and because of the suggestion that a form of atomic oxygen might serve as the attacking species in mixed-function oxidation (Staudinger, 1966).

Attention was then directed to the protein components involved in the side-chain cleavage of cholesterol. Ultrasonication of an acetone powder extract followed by chromatography on Sephadex G-200 and DEAE Sephadex A-25 resulted in the isolation of three components - an NADPH-diaphorase, a non-haem iron protein and a fraction containing cytochrome P-450. All three proteins were required to reconstitute cholesterol side-chain cleavage activity. It was found that the non-haem iron protein could only be reduced by NADPH in the presence of the diaphorase, but reduction of dichlorophenolindophenol by NADPH in the presence of the diaphorase did not require the non-haem iron protein. However, reduction of cytochrome c by NADPH was achieved only in the presence of both these proteins. This means that the sequence of electron flow is NADPH $\longrightarrow$ diaphorase $\longrightarrow$ non-haem iron protein $\longrightarrow$ cytochrome c.

Omura et al (1966) reported that the NADPH-diaphorase and the non-haem iron protein were required for NADPH-cytochrome P-450 reductase activity. In order to measure this activity, it is necessary to plot the rate of formation of the 450 mμ chromophore in the presence and absence of each of the components, as was done in the case of NADPH-cytochrome c reductase activity (fig. 53b). It was not

possible to do this, as the small changes in optical density would have necessitated using a rapid-mixing chamber and a dual-wavelength spectrophotometer. Instead, therefore, the appropriate components were simply mixed and the absorption spectrum recorded as quickly as possible. Thus it was not possible to distinguish between reaction times which were less than the time required for this operation.

Thus it was found that, using our preparation of cytochrome P-450, addition of the diaphorase was not apparently necessary to produce the 450 mμ chromophore in the P-450 difference spectrum in the presence of NADPH and carbon monoxide, only the non-haem iron protein being required; but it was not possible to demonstrate whether or not the reaction was slower in the absence of added diaphorase. All our evidence however points to the necessity of the diaphorase for the reduction of the non-haem iron protein (figs. 53 and 54). It is also necessary for NADPH-cytochrome c reductase activity. This suggests, therefore, that the cytochrome P-450 preparation was contaminated with diaphorase. Further evidence for this was the demonstration that, in reconstituted preparations which were not subjected to ultrasonication prior to fractionation, addition of the flavoprotein did not greatly stimulate cholesterol side-chain cleavage activity (Chapter 7, section 5). It was demonstrated that ultrasonication results in de-aggregation of the

P-450 preparation. This suggests that the diaphorase is normally tightly bound to the P-450 and is entrained in the aggregated P-450 preparation, resulting in incomplete separation during gel filtration. Ultrasonication prior to fractionation reduced, but did not eliminate, the contamination. The molecular sieve chromatography would not be capable of resolving aggregated and de-aggregated P-450 from one another and from P-450 contaminated with diaphorase, because all came through the column in the hole-up volume. Further evidence that two of the components were tightly bound came from a study of the relationship of rate of side-chain cleavage to concentration of unfractionated enzyme. The rate of side-chain cleavage was proportional to E^2, showing the enzyme system behaved as if it consisted of two interacting components, whereas in fact it was shown to consist of three. In their paper on the purification of the 11β-hydroxylase, Omura et al (1966) also mention the difficulty of obtaining a P-450 preparation free from contaminating diaphorase (and non-haem iron protein). Thus, it may be reasonably certain to assume that the pathway of electron flow in the cholesterol side-chain cleavage system is as in fig. 55. A feature of the reconstitution experiments was that in order to obtain activity, the isolated components had to be present at about twice the concentration that would be required to obtain the same conversion in unfractionated material. This phenomenon has been

observed in attempts to reconstitute the respiratory chain from its components (e.g. Hatefi, Haavik, Fowler and Griffiths, 1962).

The fraction containing P-450 had the typical reduced carbon monoxide difference spectrum of this cytochrome with a very small additional chromophore at 420 mμ. The haem content was calculated from the size of the 450 mμ chromophore and from the pyridine haemochromogen spectrum to be 1 mole haem/3 x 10^6g. protein. On standing at 0°C or freezing, the preparation aggregated to material with a very high particle weight, but could be de-aggregated by ultrasonication. Ultracentrifugal analysis showed that a broad spectrum of particle weights was present, in keeping with this aggregation phenomenon. The particle weight of the material at the maximum of the Schlieren peak was estimated to be very roughly 4 x 10^6. Thus, it appears that the haem group of the P-450 is bound to a particle of weight 3-4 million. This P-450 particle, although still very large, is probably smaller than any other preparation of P-450 so far reported, as it possessed excellent optical clarity to the naked eye when de-aggregated by ultrasonication, whereas other preparations (e.g. that of Cooper et al, 1965), are opaque in the absence of detergents.

Evidence that the P-450 prosthetic group of this particle is in fact the active centre of the cholesterol

side-chain cleavage system is presented in Chapters 5 and 6. In common with several other mixed-function oxidases, and more particularly the steroid 11β and 21-hydroxylases, the cholesterol side-chain cleavage system was found to be inhibited by carbon monoxide. Furthermore, the kinetics of this inhibition were consistent with a competition between oxygen and carbon monoxide for a common binding site. Such competition is a well-known feature of several haemoproteins such as cytochrome oxidase and haemoglobin. The value of the partition constant for the cholesterol side-chain cleavage system, although somewhat variable from one preparation to another was about unity, which is the same as published values for the steroid 21-hydroxylase (Estabrook et al, 1963). This value for the partition constant of mixed-function oxidases is quite different from the partition constants of haemoglobin and cytochrome oxidase which are 1.8-8.0 x 10^{-3} and 10 respectively (Keilin and Wang, 1946). It is, however, similar to the partition constant of cyto-:chrome P-450 which was calculated in parallel experiments to be 1.07 ± 0.30. This in itself suggested the possibility that cytochrome P-450 might be involved as the oxygen-binding site for the cholesterol side-chain cleavage system.

More conclusive evidence was obtained from experiments designed to derive the photochemical action spectrum for light-reversal of the carbon monoxide inhibition of the

cholesterol side-chain cleavage system. This spectrum should be very similar to the absorption spectrum of the carbon monoxide-binding centre of the system under the conditions of assay. The resulting action spectrum was very similar to the reduced carbon monoxide difference spectrum of cytochrome P-450, in that there was a pronounced maximum at 450 mμ.

However, light at 410 mμ and 490 mμ was also quite effective in reversing the carbon monoxide inhibition in some experiments. The reason for this is not understood at present. However, the carbon monoxide reduced difference spectrum of the fractionated P-450 (fig. 51a) may not represent the spectrum of the particular form of P-450 involved in side-chain cleavage, as it probably also contains a contribution from P-450 involved in 11β-hydroxylation as well. 11β-hydroxylase activity is high in acetone powder extracts, and the method of fractionation of the components of the cholesterol side-chain cleavage system did not differentiate these from the corresponding 11β-hydroxylase components. That only a small portion of the total P-450 might be involved in the cholesterol side-chain cleavage reaction was suggested by the centrifugation studies of Chapter 7, section 3, in which the 105,000 xg.-190 min. supernatant of a mitochondrial sonicate contained significant side-chain cleavage activity but no detectable P-450. However, in the case of the liver microsomal drug-metabolising

systems, it has been suggested that all these systems utilise a common pool of cytochrome P-450 and that specificity is achieved by means of a number of specific binding proteins which permit the P-450 to oxygenate the attached substrate (Orgel, 1966). A similar situation could apply in the case of adrenal cortex mitochondrial 11β-hydroxylase and cholesterol side-chain cleavage systems. Further work is needed on the purification of the mitochondrial cytochrome P-450 of adrenal cortex before this problem can be answered.

The reduced carbon monoxide difference spectrum of the fractionated P-450 (fig. 51a) showed a small chromo-:phore at 420 mμ when the preparation was reduced with dithionite, but not when reduced with the NADPH-cytochrome P-450 reductase (fig. 54a). The latter conditions were capable of reducing haemoglobin to produce the Soret band of carboxyhaemoglobin, so it was concluded that the 420 mμ absorption was due entirely to P-420 which consequently did not appear reduceable by the NADPH-cytochrome P-450 reductase. Consequently, in unfraction-:ated preparations which possessed a large 420 mμ chromo-:phore in the NADPH-reduced carbon monoxide difference spectrum, such as supernatant of mitochondrial sonicate (fig. 18), this must have been due to contaminating haemoglobin.

The non-haem iron protein had an absorption spectrum which was very similar to that of 'adrenodoxin' of

Kimura and Suzuki (1967), and was bleached in air on addition of dithionite or NADPH plus diaphorase. Thus, unlike some other similar proteins, this non-haem iron protein did not seem to be readily autoxidiseable. This is in agreement with the findings of Omura et al (1966) for adrenodoxin. The protein contained acid-labile sulphur, as shown by the odour of hydrogen sulphide on addition of dilute HCl. The iron content was estimated to be 1 g.atom Fe/12,000 g. protein. Kimura and Suzuki (1967) consider that adrenodoxin contains 2 atoms of iron per molecule, and that the molecular weight is about 19,000, suggesting our non-haem iron protein is somewhat impure. That this was so was shown by electrophoresis on cellulose acetate strips and in polyacrylamide gel, which revealed perhaps three protein contaminants. However, the characteristic brown colour of the non-haem iron protein was confined to only one of the bands. No attempt has been made to purify this protein rigorously, one of the problems in such an attempt being the instability of the purified protein, which was observed to bleach considerably during ion-exchange chromatography. However, we have obtained no information to suggest that the non-haem iron protein isolated as described here differs in any way from the adrenodoxin of Kimura and Suzuki (1967) and of Omura et al (1966) which is a component of the 11β-hydroxylase, nor have we any evidence to suggest that there are two non-haem iron proteins in our preparation,

one for cholesterol side-chain cleavage, and one for 11β-hydroxylation. In this connection it is interesting to note that the elution pattern on the DEAE-Sephadex obtained here (fig. 45b) is practically identical with that of 150,000 xg.-100 min. supernatant of mitochondrial sonicate on DEAE-cellulose, obtained by Omura et al (1966).

The NADPH-diaphorase was a bright yellow colour which was bleached by dithionite or NADPH in air, showing that this protein also was not readily autoxidiseable. The prosthetic group had an absorption spectrum typical of a flavin, and paper chromatography of a sample showed it had an R_F similar to FAD. Electrophoresis of a sample of the diaphorase on cellulose acetate showed one major protein band, coinciding with the yellow colour of the flavin.

Thus, this work has served to throw some light on the mechanism of cholesterol side-chain cleavage in adrenal cortex, and provides material for speculation on the behaviour of the system in the whole cell. Brownie and Grant (1954) showed that 11β-hydroxylation in bovine adrenal cortex mitochondria was supported by Krebs cycle intermediates such as α-ketoglutarate, succinate and malate. Inhibition by the uncoupling agent dinitrophenol suggested that 11β-hydroxylation was dependent on oxidative phosphorylation. Harding et al (1965) showed for rat adrenal mitochondria that succinate or malate were more

effective in supporting 11β-hydroxylation than was exogenous NADPH. With succinate, respiratory chain inhibitors such as amytal abolished 11β-hydroxylation. This suggested to Harding and Nelson (1966) that reduction of $NADP^+$ by succinate might occur via reversed electron transport in the NADH dehydrogenase region of the respiratory chain. They also proposed that the reduction of $NADP^+$ by NADH may occur via an energy-linked trans-:hydrogenase. Similar suggestions have been made by Péron, McCarthy and Guerra (1966) and Guerra, Péron and McCarthy (1966).

A similar situation has been found by Koritz (1966) in the case of the cholesterol side-chain cleavage system, which in whole mitochondria also utilises succinate more effectively than exogenous NADPH. Succinate-supported side-chain cleavage was inhibited by amytal and antimycin but not oligomycin. This suggests that in the cholesterol side-chain cleavage system also, succinate supplies electrons via reversal of electron transport. In further support of this, Hall (1967) found succinate-supported side-chain cleavage of cholesterol to be inhibited by hyperbaric oxygen, which is a specific inhibitor of reverse electron transport (Chance, Jamieson and Coles, 1965).

Thus, the cholesterol side-chain cleavage system and the 11β-hydroxylase appear to be identical to one

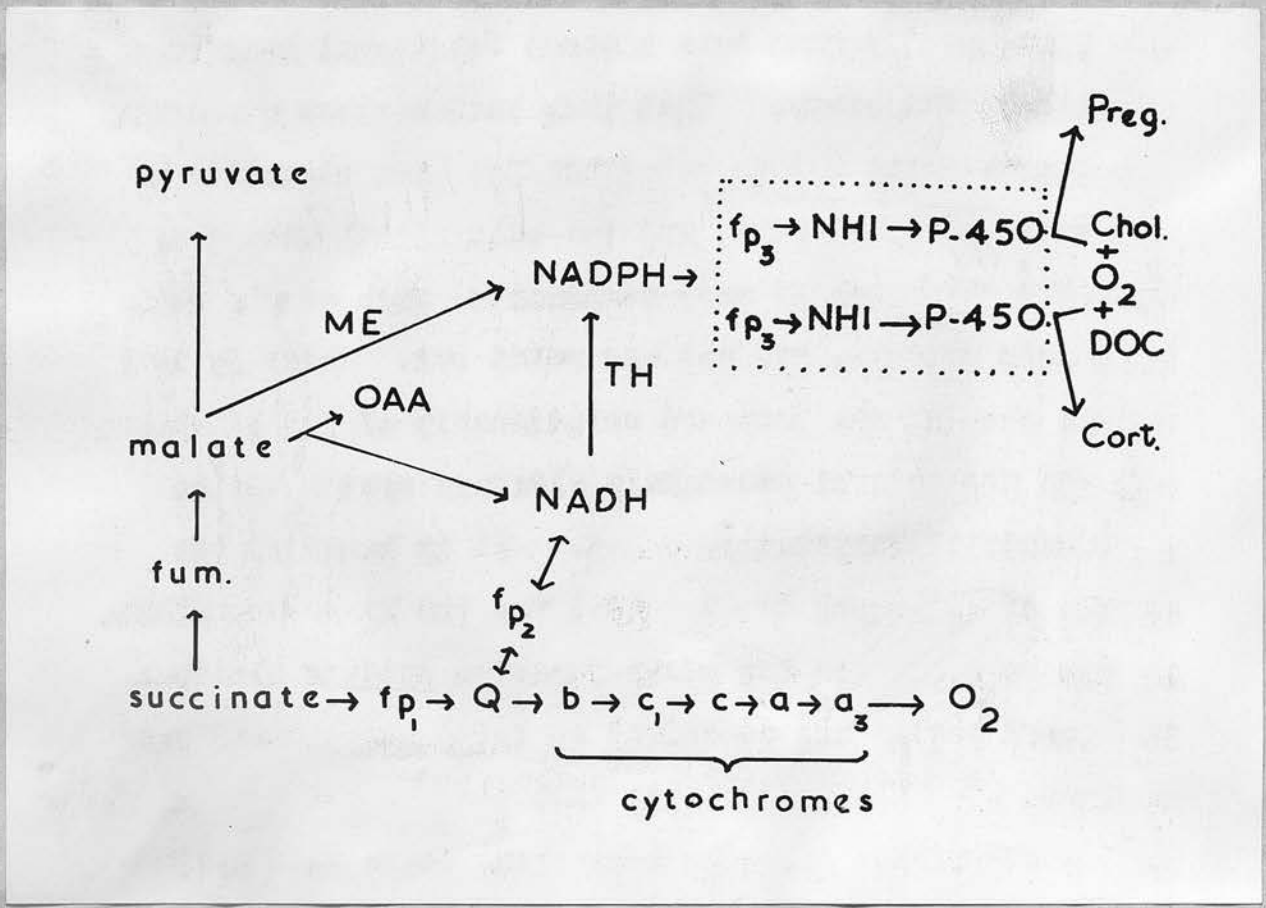


Fig.57. Proposed relationship of cholesterol side-chain cleavage system and 11β-hydroxylase system to one another and to the mitochondrial respiratory chain. The dotted lines indicate the possibility that the two systems may share one or more components.

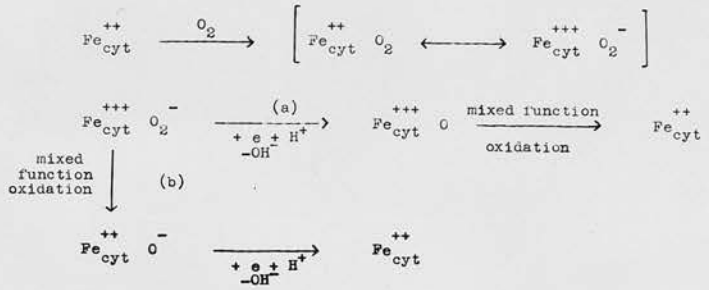
OAA - oxaloacetate. TH - transhydrogenase.

ME - malic enzyme. chol. - cholesterol.

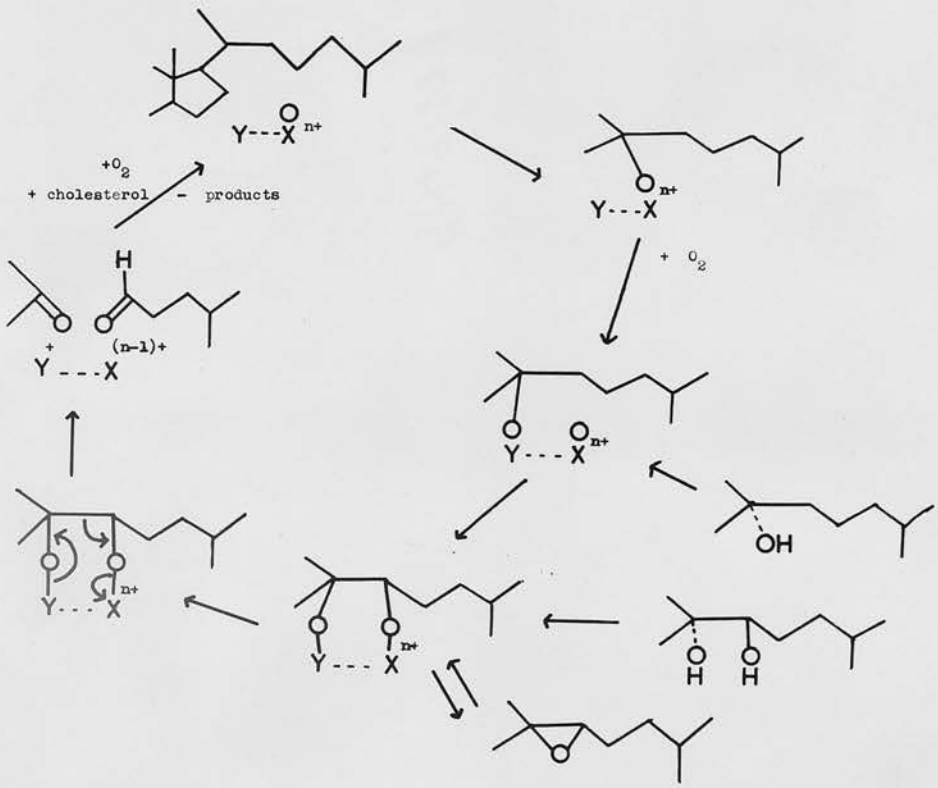
preg. - pregnenolone. cort. - corticosterone.

fp - flavoprotein. NHI - non-haem iron protein.

another in their relationship to the respiratory chain, and consequently must have a close functional relationship the one to the other. That they bear a close structural relationship the one to the other has been suggested by the work described here, and the possibility that they may even share one or more components, such as the non-haem iron protein, has not been ruled out. Fig. 57 is a scheme showing the proposed relationship of the 11β-hydroxylase and the cholesterol side-chain cleavage system to the mitochondrial respiratory chain. It is based on the scheme of Estabrook et al (1966) for the 11β-hydroxylase. As can be seen, the two mixed-function oxidase electron transport chains are conceived as lying parallel to one another, with the possibility that the two systems may share common components shown by encircling these with dotted lines.



Let $X^{n+}O \equiv Fe^{+++}_{cyt}\ O_2^-$ or $Fe^{+++}_{cyt}\ O$



Hypothetical scheme of cholesterol side-chain cleavage.

Fe_{cyt} - haem iron of cytochrome P-450.

Y - second ligand, either a second haem iron of P-450, or another metal group, or a sulphydryl, or $-H$.

Fig. 58.

A Possible Mechanism of Cholesterol Side-chain Cleavage

This work has made it possible to speculate on the mechanism of cholesterol side-chain cleavage in the adrenal cortex, and to suggest a reaction sequence which differs from the traditional pathway involving free 20α-hydroxy-:cholesterol and 20α,22ξ-dihydroxycholesterol. This is shown in fig. 58.

In this scheme the involvement of a metal which is likely to be the haem iron of cytochrome P-450, is implied. The question as to whether or not the attacking oxygenated species contains both atoms of each oxygen molecule, or just one as suggested by Staudinger (1966), is left open. Also undecided is the problem as to whether there are separate enzyme sites for oxygen insertion at C_{20} and C_{22}, or whether one site is responsible for oxygen insertion in both. In this mechanism, the cleavage of the cholesterol side-chain is somewhat analogous to the lead tetraacetate cleavage of glycols. 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol could enter the reaction sequence as 'substrates' at the points indicated. The role of electrons from the NADPH would be to reduce the remaining atom of each oxygen molecule to water, whether this occurred before oxygen insertion, as in the mechanism of Staudinger (1966), or after.

For the future, it will be necessary to rigorously purify the components of the cholesterol side-chain

cleavage system, particularly the cytochrome P-450 fraction. When this has been achieved, it will be possible to establish the relationship between the cholesterol side-chain cleavage system and the 11β-hydroxy-:lase system. It seems quite possible that the two systems will be found to share common 'pools' of flavoprotein and non-haem iron protein, with specificity residing in the cytochrome P-450 fraction. Two alternatives can be envisaged here - either separate forms of P-450 for each of the two mixed-function oxidases, or alternatively, a common 'pool' of P-450 shared by both systems, and specific binding proteins, one for cholesterol and one for 11β-hydroxylase substrates, which permit the P-450 to oxygenate the attached substrate, as suggested by Orgel (1966) for liver microsomal mixed-function oxidases.

The relevance of this relationship to the overall pattern of steroidogenesis in adrenal cortex is not immediately obvious, in view of the fact that the cholesterol side-chain cleavage reaction is the first step in the catabolism of cholesterol, and must therefore be a key reaction in the control of hormone formation. Other steroid hydroxylases do not apparently share this relationship, for example the 21-hydroxylase is located in the endoplasmic reticulum and consequently does not possess the same links with the mitochondrial respiratory chain shown for the other two systems. Control mechanisms such as availability of electrons from energy

sources and factors affecting protein synthesis must be different in the case of mitochondrial and microsomal systems. The evidence that mitochondria are capable of self-replication (reviewed by Lehninger, 1964), may point to independent factors affecting biosynthesis of mitochondrial mixed-function oxidases, when contrasted with microsomal systems.

These matters are components of the overriding problem in adrenal steroidogenesis, namely its control by ACTH. This control could be applied at any or all of three levels - control of enzyme activity by alteration of the kinetic parameters of the enzymes; control of availability of reducing equivalents; and control of the rate of enzyme synthesis. Complete elucidation of the enzymology of these mixed-function oxidase reactions is extremely relevant to a detailed understanding of these control processes, not only in adrenal cortex but also in the other endocrine tissues under the influence of the anterior pituitary.

APPENDIX 1

Materials

The following chemicals were purchased from the Sigma Chemical Co.:-

$NADP^+$ monosodium salt
FAD disodium salt
FMN sodium salt
G-6-P disodium salt
G-6-P dehydrogenase
2,6-dichlorophenol indophenol
4,7-diphenyl-1,10-phenanthroline sulphonate sodium salt (bathophenanthroline sulphonate)

Reduced glutathione was purchased from Koch-Light Laboratories Ltd., Colnbrook.

PPO scintillation grade, and POPOP, scintillation grade, were purchased from the Packard Instrument Co. Inc.

Hyamine (p-(diisobutyl-cresoxyethoxyethyl) dimethyl benzyl ammonium hydroxide) was purchased from the Packard Instrument Co. Inc.

Cholesterol-4-^{14}C and cholesterol-26-^{14}C, specific activity 60 µc./mg., were purchased from the Radiochemical Centre, Amersham.

Sephadex G-100 and G-200, and DEAE-Sephadex A-25, were purchased from Pharmacia, Uppsala.

Polyethylene glycol (Carbowax 20-M) was purchased from Union Carbide Chemical Co.

Silica gel H was purchased from Merck.

Phosphate buffer salts, potassium chloride, sodium chloride, magnesium sulphate, sucrose and ferrous ammonium sulphate hexahydrate were analar reagent grade chemicals.

Ammonium sulphate was B.D.H. enzyme grade, specially low in heavy metals.

All other reagents and solvents were analar reagent grade, with the exception of toluene, ethanol, methanol and acetone. Toluene was washed with conc. sulphuric acid and water, and dried over sodium sulphate. Other solvents were purified as described in the text.

20α-hydroxycholesterol was synthesised by the method of Petrow and Stuart-Webb (1956).

22,24,25 and 26-hydroxycholesterols were synthesised in this laboratory by Mr. S.A.M. Ali.

20α,22R-dihydroxycholesterol was a gift from Dr. R.I. Dorfman.

APPENDIX 2

Instruments

Radioactivity measurements were performed in a Packard Tri-Carb 314EX liquid scintillation spectrometer. The scintillation liquid consisted of 4 g./litre PPO and 30 mg./litre POPOP in toluene.

Radioactivity measurements were also performed using a thin-layer radioactive scanning device, constructed in this department by Mr. A. Purdie. The design was based on that of Ravenhill and James (1966). The gas mixture was argon containing 5 per cent carbon dioxide and counting was in the proportional range. This instrument gave an absolute counting efficiency of 25 per cent for carbon-14 on thin-layer plates 0.25 mm. thick.

Optical measurements were performed in an 'Optica' CF4DR double beam recording spectrophotometer, and a 'Unicam' S.P.600.

Ultrasonic disintegration of mitochondria was performed using an M.S.E.-Mullard ultrasonic disintegrator operating at 20 Kc./sec.

Protein fractionation was performed using an L.K.B. 7000A "UltroRac" fraction collector. Extinction at 280 mμ was monitored by means of an L.K.B. 8300A Uvicord II Absorptiometer and 6520 H recorder.

Gas-liquid chromatography was performed on a Pye Argon Chromatograph with a radioactivity detection system based on that of James and Piper (1961).

Interference filters with transmission maxima at 403, 410, 418, 429, 448, 456, 470, 491 and 502 mμ were purchased from Grubb Parsons Ltd., Newcastle-upon-Tyne. The average half band width was 4-5 mμ.

APPENDIX 3

Analytical Procedures

Protein Concentration was measured by the Biuret method of Layne (1957) using bovine serum albumen as standard.

Acid-extractable Iron This was measured by a modification of the method of Massey (1957) and Ramsey (1958). 1.2 ml. aliquots were made up to 1.5 cm. with conc. hydrochloric acid to give an acid concentration of 2.2N. After standing a further 10 min. the samples were centrifuged and the supernatants decanted. To each was added 1 ml. pyridine followed by o.1 ml. ascorbic acid solution (5 mg./ml. distilled water). After standing 10 min. the tubes were read at 535 mμ using a 'Unicam' S.P.600. The acid-extractable iron was taken as a measure of non-haem iron.

Acid-nonextractable Iron The protein precipitates from the above determinations were incubated 30 min. in a boiling water bath with 0.1 ml. hydrogen peroxide and 0.1 ml. per chloric acid. After allowing to cool somewhat, 0.1 ml. 10 per cent aqueous hydroxylamine was added. After 5 min., 0.1 ml. bathophenanthroline sulphonate solution was added. After 10 min. the tubes were read at 535 mμ. This is a modification of the method of Adler and George (1965). The acid-nonextractable iron was taken as a measure of haem iron.

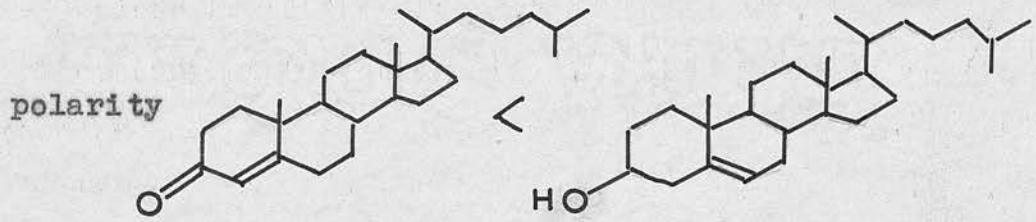
These iron determinations were made quantitative where necessary from a calibration graph using freshly prepared ferrous ammonium sulphate as standard. All glassware for iron determinations was soaked in strong hydrochloric acid and washed thoroughly with distilled water before use.

NADPH-diaphorase To 2 ml. aliquots was added 0.02 ml. NADPH-generator (7.5 mg. $NADP^+$; 25 mg. glucose-6-phosphate; 1 unit glucose-6-phosphate dehydrogenase per ml. water), followed by 0.02 ml. of a 3 mg./ml. aqueous solution of 2,6-dichlorophenol indophenol. After 5 min. the decrease in absorbency at 590 mμ was read. This is based on the method of Omura et al (1966).

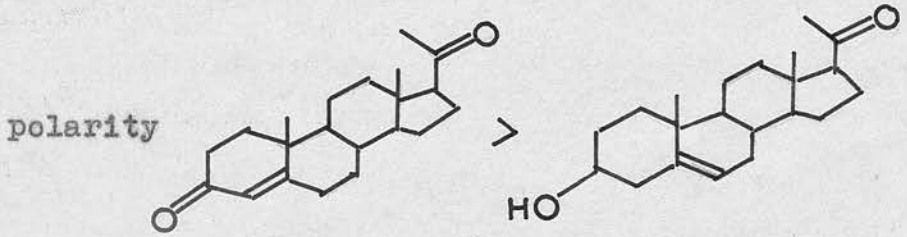
APPENDIX 4

Relative polarities of pregnenolone and progesterone

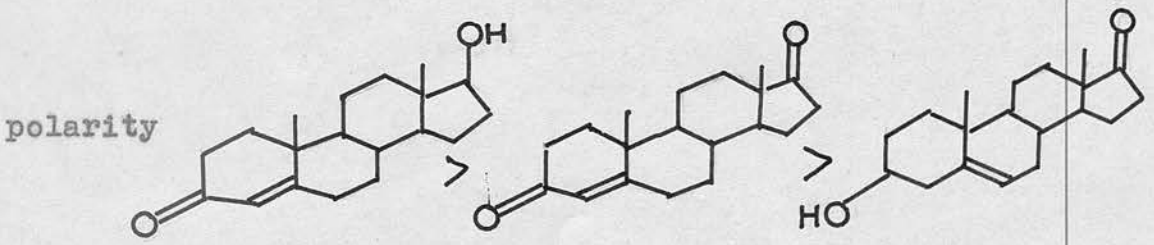
In most solvent systems for T.L.C., steroids with the Δ^4-3-one structure are less polar than the corresponding steroid with the Δ^5-3β-ol structure. In the solvent systems A and B, i.e. those consisting of petroleum, acetic acid and an ether, this is so in the case of cholesterol and cholest-4-ene-3-one, i.e.



However, in the case of pregnenolone and progesterone, the polarities were found to be reversed:-



A similar situation was found in the case of dehydroepiandrosterone and androst-4-ene-3,17-dione:-



testosterone androstene-:dione dehydroepi-:androsterone

This effect of reversal of polarity would therefore appear to be a function of steroids possessing Δ^4-3-one or Δ^5-3β-ol structures which also have a 20-one or a 17-one function.

APPENDIX 5

Detection of non-radioactive steroids on thin-layer plates

Steroids were detected by spraying the plate with one of several solutions:-

(a) Phosphomolybdic acid in ethanol. This non-specific reagent shows up many organic compounds including most steroids as dark blue spots against a yellow background, after heating the plates at 120°C for a few minutes. Steroids containing the Δ^4-3-one structure, however, require a longer period of heating and even then the colour is faint. The same applies to 7-oxo cholesterol.

(b) Phosphotungstic acid in ethanol. With this reagent, many steroids display a highly specific colour against a white background, again after heating; some of these colours are listed below.

Steroid	Colour immediately after spraying	after four hours
cholesterol	orange	pink
cholesterol 3β-acetate	orange	pink
pregnenolone	orange	pink
pregnenolone 3β-acetate	orange	pink
20α-hydroxycholesterol	green with pink rim	grey
20α-hydroxycholesterol 3β-acetate	green with pink rim	grey
20α,22R-dihydroxy-cholesterol	grey	grey-blue

Steroid	Colour immediately after spraying	after four hours
7α-hydroxycholesterol	deep blue	deep blue
7β-hydroxycholesterol	deep blue	deep blue

Steroids with a Δ^4-3-one structure do not give any colour with this reagent, and neither does 7-oxo cholesterol.

(c) Scintillation liquid diluted 1:4 with methanol.

When viewed under an ultraviolet lamp, T.L.C. plates sprayed with this solution show organic compounds not containing double bonds as pale blue against a darker blue background. Compounds with conjugated double bonds, such as steroids with the Δ^4-3-one structure or 7-oxo cholesterol, show up as dark spots.

Ultraviolet-absorbing steroids could also be displayed by incorporating an inorganic phosphor, such as Willemite, into the silica gel to the extent of 0.5 per cent. When viewed in ultraviolet light, such steroids showed up as dark spots against a bright green background.

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Studies on the Side-chain Cleavage of Cholesterol by Bovine Adrenal Cortical Mitochondria

By E. R. Simpson and G. S. Boyd. (*Department of Biochemistry, University of Edinburgh*)

Studies by Estabrook, Cooper & Rosenthal (1963) and Cooper, Rosenthal, Foroff, Slade & Levin (1965) have shown the inhibitory effects of carbon monoxide on two mixed-function oxidases involved in steroid metabolism, namely the 21-hydroxylase of adrenal-cortex microsomes, and the 11β-hydroxylase of adrenal-cortex mitochondria. In each case inhibition by carbon monoxide correlated with the appearance of an absorption maximum at 450mμ in the reduced difference spectrum. This carbon monoxide-binding pigment, a haemoprotein, is believed responsible for oxygen activation in these hydroxylation reactions (Omura, Sato, Cooper, Rosenthal & Estabrook, 1965). Since the enzyme system catalysing the cleavage of the cholesterol side-chain in adrenal cortical mitochondria has the properties of a mixed-function oxidase, the effect of carbon monoxide on this system was investigated.

Bovine adrenal cortical mitochondria prepared in the usual way, were sonicated in hypotonic medium in an M.S.E.-Mullard Ultrasonic Disintegrator for 15min. at 0°. Centrifugation of the sonicate for 30min. at 105000 *g* gave an opalescent supernatant containing the enzymic activity. Incubations were conducted at 37° for 15min. in the presence of an NADPH-generating system, magnesium sulphate and phosphate buffer, pH 7·4, using [4-^{14}C]cholesterol (Amersham) as substrate. The products were analysed by thin-layer chromatography, using a gas-flow thin-layer scanner and also liquid scintillation spectrometry in a Packard Tri-Carb spectrometer. In these studies, the major product was pregnenolone, with a trace of progesterone. No products which might be considered as intermediates between cholesterol and pregnenolone were detected. Optical measurements were performed in an 'Optica' double-beam recording spectrophotometer with cuvettes modified to permit the bubbling of appropriate gas-mixtures through the solutions.

In the presence of 10% oxygen, carbon monoxide produced a peak at 450mμ in the reduced difference spectrum. This peak increased rapidly with increasing percentages of carbon monoxide, reaching a maximum at 40% carbon monoxide. Increase in peak size was paralleled by a corresponding proportionate decrease in cholesterol side-chain cleavage activity. Maximum inhibition was achieved at 40% carbon monoxide content. Harding, Wilson, Wong & Nelson (1965) obtained similar results in the case of the steroid 11β-hydroxylase. These findings indicate that the mechanism of oxygen activation in the cholesterol side-chain cleavage system is similar to that involved in other mixed-function oxidases involved in steroid metabolism.

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THE CHOLESTEROL SIDE-CHAIN CLEAVAGE SYSTEM OF THE ADRENAL CORTEX: A MIXED-FUNCTION OXIDASE

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In the adrenal cortex, cholesterol is metabolized to the corticosteroid hormones. The first step in this process is the cleavage of the cholesterol side-chain to form pregnenolone, proposed intermediates being 20α-hydroxycholesterol (Shimizu, Hayano, Gut and Dorfman, 1961; Constantopoulos and Tchen, 1961) and 20α,22-dihydroxycholesterol (Shimizu, Gut and Dorfman, 1962; Constantopoulos, Satoh and Tchen, 1962). The enzyme system catalysing the cholesterol side-chain cleavage occurs in adrenal cortex mitochondria and has a requirement for molecular oxygen and reduced NADP (Halkerston, Eichhorn and Hechter, 1961); thus it has the characteristics of a mixed-function oxidase (Mason, 1957).

Studies on two other mixed-function oxidases of the adrenal cortex namely the steroid 11β- and 21-hydroxylases have shown these systems to be inhibited by carbon monoxide, this inhibition being correlated with the appearance of an

absorption maximum at 450 mμ. in the reduced difference spectrum (Estabrook, Cooper and Rosenthal, 1963; Harding, Wilson, Wong and Nelson, 1965). This carbon monoxide-binding pigment, a haemoprotein, is believed responsible for oxygen activation in these hydroxylation reactions, and has been designated 'P-450' (Omura and Sato, 1962).

In order to see if a similar situation applies in the case of the cholesterol side-chain cleavage system, the effects of carbon monoxide on this system were investigated. The evidence presented in this paper suggests that 'P-450' is indeed a component of the cholesterol side-chain cleavage system.

Methods

Bovine adrenal glands were obtained fresh from slaughter. The cortices were removed, chopped finely, and homogenised in 3 volumes of ice-cold 0.25M sucrose using an all-glass homogeniser. The mitochondria were isolated in the usual way, and washed twice with 0.154M KCl. The washed mitochondrial pellet was suspended in distilled water and sonicated as suggested by Cooper, Narasimhulu, Slade, Raich, Foroff and Rosenthal (1965) for 15 min. at 0° C with an M.S.E. Mullard Ultrasonic Disintegrator operating at 20 Kc./sec. Centrifugation of the sonicate at 105,000 x g for 30 min. gave an opalescent supernatant containing the enzymic activity. This activity was assayed by incubating cholesterol-4-C^{14}, purchased from the Radiochemical Centre, Amersham, and purified by thin-layer chromatography immediately before use, with the sonicate in the presence of an NADPH-generating system

buffered at pH 7.4 with phosphate (Table 1). Incubations were performed at 37° C for 15 min. in optical cuvettes modified to permit the bubbling of appropriate gas mixtures through the solutions. The reactions were stopped with methanol and the extracted sterols separated by thin-layer chromatography on silica gel H (Fig. 1). Analysis of the products was achieved by means of a gas-flow thin-layer radioactive scanner, and liquid

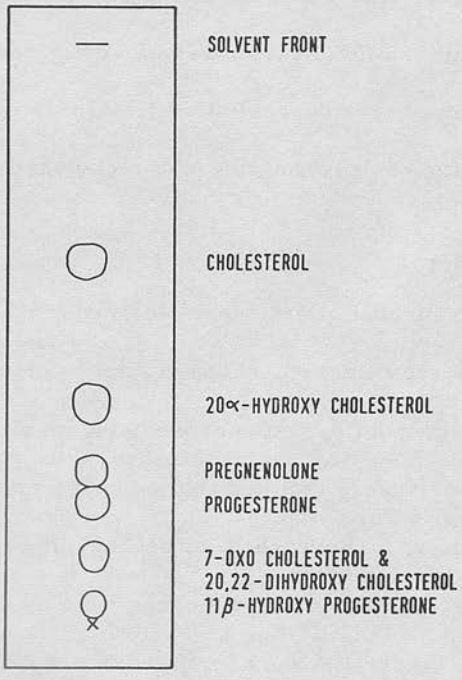

Fig. 1. Thin-layer plate showing separation of typical sterols. The plates were run in the solvent system petroleum ether: diethyl ether:acetic acid 75:25:2 until the solvent front was three-quarters of the plate length from the origin. The plates were then re-run in the solvent system petroleum ether:diethyl ether: acetic acid 65:35:2 until the solvent front reached the top of the plate.

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scintillation spectrometry using a Packard Tri-Carb 314 EX.

Optical measurements were performed in an 'Optica'

CF4DR double-beam recording spectrophotometer at room temperature using the modified optical cells. Fig. 2 shows the reduced difference spectrum of the enzyme preparation in the presence of carbon monoxide. The 420 mμ. absorption may be due in part to contaminating haemoglobin which it was not possible to remove from these preparations. The 450 mμ. chromophore is characteristic of the 'P-450' pigment.

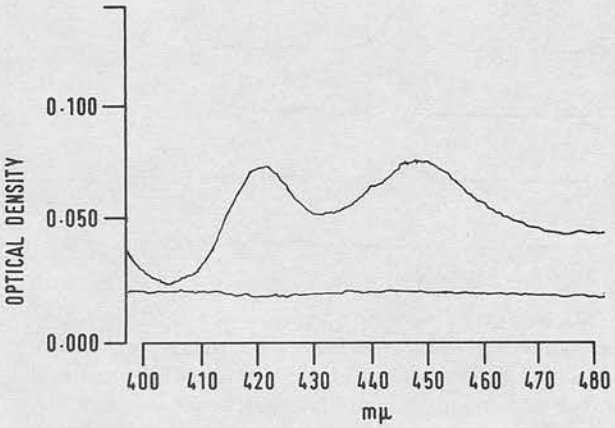

Fig. 2. Reduced difference spectrum. For the base line, each cell contained the incubation mixture shown in Table 1 but without the cholesterol-4-C^{14}. When carbon monoxide was bubbled through the experimental cell, the characteristic difference spectrum appeared.

Results and Discussion

In the presence of 10% oxygen, increasing the percentage carbon monoxide in the gas mixture produced a rapid increase in the size of the 450 mμ. absorption band. Maximum peak size was attained at 40% carbon monoxide.

When the side-chain cleavage activity was assayed in the presence of 10% oxygen and increasing proportions of carbon monoxide, the results shown on Fig. 3 were obtained. In the radioactive assays the peak on the left is the added cholesterol

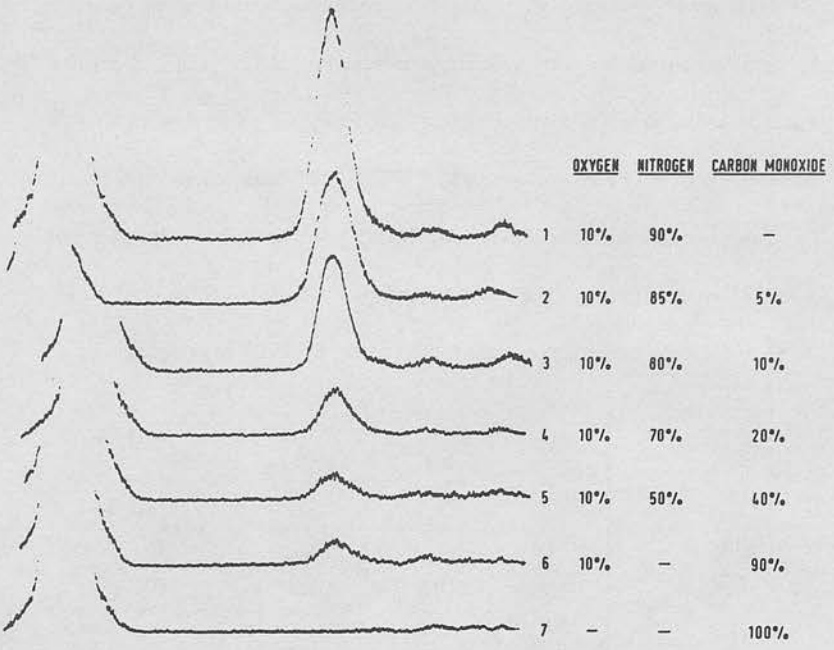

Fig. 3. Radioactive scanner traces of the thin-layer plates under the conditions shown in the accompanying Table. The peak on the left is the added cholesterol substrate and the peak in the centre the major product, pregnenolone.

substrate, and the major product is pregnenolone. There is a trace of progesterone and some 11β-hydroxyprogesterone. Some 7-oxocholesterol (formed autoxidatively) is also present. No labelled 20α-hydroxycholesterol or 20α,22-dihydroxycholesterol have been identified in these incubations. The pregnenolone peak represents a conversion of some 15% of the added radioactive cholesterol when the gas phase is 10% oxygen and 90% nitrogen, under the conditions given.

Fig. 4 shows a graphical presentation of the results of Fig. 3 together with the variation in size of the 450 mμ.

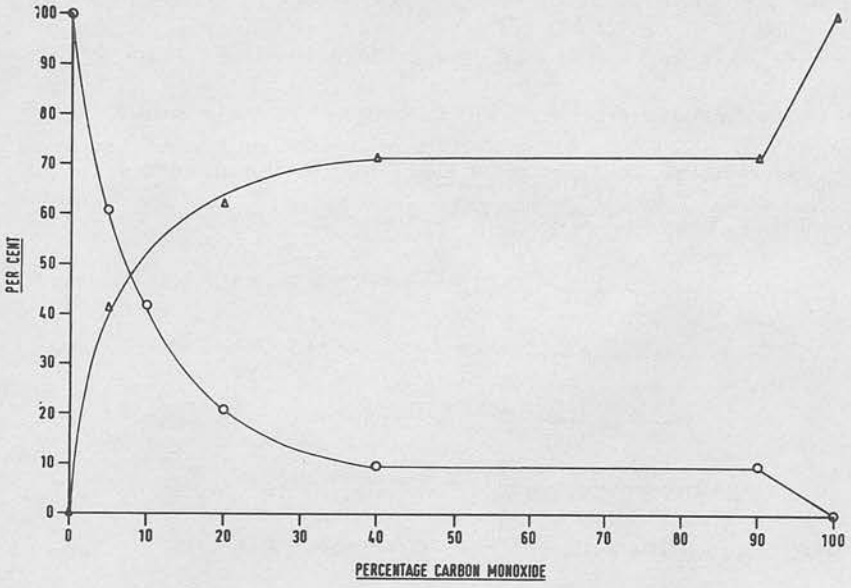

Fig. 4. O - Enzymic activity (percentage of that occurring in the absence of CO).
Δ - Height of 450 mμ. peak (percentage of that obtained in 100% CO).
Graph of enzymic activity and height of 450 mμ. chromophore versus percentage carbon monoxide. The oxygen content was constant at 10% in all studies except when the gas phase was 100% carbon monoxide.

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absorption band with increasing carbon monoxide content. As the 'P-450' peak increases in size, there is a corresponding decrease in the side-chain cleavage activity. With 10% oxygen in the gas phase, maximum inhibition and maximum size of the 'P-450' peak were attained at 40% carbon monoxide content. With 100% carbon monoxide there is complete inhibition of side-chain cleavage activity and a further increase in size of the 'P-450' peak. This implies that the carbon monoxide binding, which gives rise to the 450 mμ. chromophore, is responsible

for the inhibition of the side-chain cleavage activity. Thus 'P-450' is implicated as a component of the cholesterol side-chain cleavage system. These results are quite similar to those obtained by Harding et al. (1965) for the steroid 11β-hydroxylase.

Table 1

Incubation Mixture

SONICATE SUPERNATANT	15 mg. protein/ml.	1 ml.
PHOSPHATE BUFFER	0.1M pH 7.4	1.2 ml.
MAGNESIUM SULPHATE	200 μ moles/ml.	0.125 ml.
NADP (in distilled water)	37.5 mg./ml.	0.05 ml.
G-6-P (in distilled water)	125 mg./ml.	0.05 ml.
G-6-P DEHYDROGENASE	1 unit/0.1 ml.	0.05 ml.
CHOLESTEROL-4-C^{14}	0.25 μ C in 0.05 ml. ACETONE	0.05 ml.

This 'P-450' pigment although accepted as being responsible for oxygen activation in several steroid mixed-function oxidases has not yet been isolated in a pure form. It is believed to be a cytochrome, and in keeping with other cytochrome systems responsible for oxygen activation, it would be expected to lie at the end of an electron transport pathway. That this is so in the case of the steroid 11β-hydroxylase has been shown by Omura and co-workers (Omura et al. 1965). In this, electrons pass from the reduced pyridine nucleotide

to the 'P-450' reaction centre via an electron transport pathway consisting of flavoprotein and a non-haem iron protein. We tentatively suggest a similar scheme for the cholesterol side-chain cleavage system.

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Steroid Hydroxylation in the Adrenal Cortex and the Cholesterol Side-Chain Cleavage System

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Carbon monoxide inhibition and its reversal by light are well-known characteristics of several haem-containing proteins involved in oxygen binding. Studies on several mixed-function oxidases have revealed that these are also inhibited by carbon monoxide, for example the steroid 21-hydroxylase of the adrenal cortex (Ryan & Engel, 1957) and several drug-hydroxylating systems of liver microsomes (Cooper, Levin, Narasimhulu & Rosenthal, 1965). The carbon monoxide inhibition of these systems is light-reversible and the most effective wavelength is 450mμ (Omura, Sato, Cooper, Rosenthal & Estabrook, 1965). This has been interpreted to imply that a haemoprotein with an absorption peak at 450mμ in the carbon monoxide reduced difference spectrum is responsible for oxygen binding in these systems.

The first step in the catabolism of cholesterol in the adrenal cortex is the cleavage of the cholesterol side chain to form pregnenolone. We have demonstrated that this system is inhibited by carbon monoxide (Simpson & Boyd, 1966).

The enzyme source was a sonicate of bovine adrenal-cortex mitochondria. [4-^{14}C]Cholesterol was the substrate and the assay was based on the conversion to pregnenolone. Incubations were performed at 37° in the presence of an NADPH-generating system buffered at pH 7·4 with phosphate. The pregnenolone was isolated and estimated using thin-layer chromatography and liquid-scintillation spectrometry.

The partition constant for the carbon monoxide binding was found to vary between 0·2 and 1·2 depending on the mitochondrial preparations. This is in agreement with the published values for the microsomal steroid 21-hydroxylases (Estabrook, Cooper & Rosenthal, 1963).

The carbon monoxide inhibition of the side-chain cleavage activity was investigated for light-reversibility. Using a high-pressure xenon lamp and a series of interference filters it was found that the most effective wavelength for light reversal was 450mμ, while in some preparations 410mμ and 490mμ were also partially effective.

This suggests that the haemoprotein implicated as the terminal oxidase of several mixed-function oxidases is a component of the side-chain cleavage system also. However the secondary peaks at 410 and 490 mμ in the photochemical action spectrum may indicate that the situation in the case of the cholesterol side-chain cleavage system is more complex. These problems can only be settled by the complete resolution of the system into its many components and work on this aspect is now in progress in this Laboratory.

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